Development of a Chemiluminescent Assay for Autoantibodies to Thyroid Peroxidase

by

Karen Catherine Thomas

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Contents

Certificate of Research

This is to certify that, except where specific reference is made, the work described in this thesis is the result of the candidate. Neither this thesis, nor any part of it, has been presented, or is currently submitted, in candidature for any degree at any other University.

Signed ...K. C. Thomas...
Candidate

Signed .....
Director of Studies

Date .....

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My final thanks go to my parents to whom this thesis is dedicated.

Abbreviations Used in Text

All the abbreviations [apart from standard chemical formulae and *Systeme Internationale* (S.I.) units], found within the text are listed below.

ABTS	2-2' azino-di-(3-ethyl benzothiazolin sulphone-6 diammonium salt)
AE	Acridinium ester
Acrid-Anti-IgG	Anti-human IgG labelled with acridinium ester
Acrid-IgG	IgG (containing anti-TPO) labelled with acridinium ester
Acrid-Tg	Tg labelled with acridinium ester
Acrid-TPO	Thyroid Peroxidase labelled with acridinium ester
α-IFN	Alpha-interferon
AITD	Autoimmune thyroid disease
A_{MAX}	Maximum absorbance
ANA	Antinuclear antibody
Anti-ssDNA	Anti single-stranded Deoxyribonucleic Acid
APS	Autoimmune polyglandular syndrome
Biotin-TPO	Biotinylated/TPO label
BL	Bioluminescence
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
CF	Complement fixation
CIA	Chemiluminescent immunoassay
CL	Chemiluminescence
CNBr	Cyanogen bromide
CS	Cyclosporin
C.V.	Coefficient of Variation
ddH_2O	Double-distilled water

DIT	Di-iodotyrosine
DM	Diabetes mellitus
DOC	Deoxycholic acid
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra-acetate
EIA	Enzyme-immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
Fc	Constant fragment (of Ig)
GD	Graves' disease
HAT	Hypothyroid autoimmune thyroiditis
HLA	Human Leucocyte Antigen
HRP-TPO	Horseradish Peroxidase/TPO label
HT	Hashimoto's thyroiditis
^{125}I	Radioactive iodine
Ig	Immunoglobulin
ILMA	Immunochemiluminometric assay
IMF	Indirect Immunofluorescence
InAg	Indirect Agglutination
IRMA	Immunoradiometric Assay
kDa	Kilodaltons
LATS	Long Acting Thyroid Stimulator
MAb	Magnetic antibody
MAb-Anti-IgG	Magnetic particles coupled to human anti-IgG
MHC	Major Histocompatibility Complex
MHD	Minimum haemolytic dose
MicAb	Microsomal autoantibody

MIT	Mono-iodotyrosine
MPA	Magnetic particles coupled to Protein A (*Staphylococcus aureus*) i.e. Protein A Suspension
MS	Multiple sclerosis
Mr	Molecular mass
MWt	Molecular weight
NHS	N-hydroxysuccinimidyl
OD	Optical density
OPD	ortho-phenylenediamine dihydrochloride
PA	Pernicious anaemia
PBS	Phosphate-buffered saline
PH	Passive Haemagglutination
PMT	Photo-multiplier tube
PPT	Post-partum thyroiditis
Q.C.	Quality Control
RA	Rheumatoid arthritis
RBC	Red blood cell
RIA	Radioimmunoassay
RLU	Relative Light Units
R.O.C.	Receiver Operator Characteristic
r.p.m.	Revolutions per minute
rT_3	Reverse tri-iodothyronine
S.D.	Standard deviation of the mean
S.E.	Standard error of the mean
SLE	Systemic lupus erythmatosis
SMA	anti-Smooth-muscle antibody
SpA	Protein A

SRID	Single radial immunodiffusion
T_3	Tri-iodothyronine
T_4	Thyroxine
TBII	TSH Binding Inhibitory Immunoglobulin
TBS	Tris buffered saline
Tg	Thyroglobulin
TgAb	Thyroglobulin autoantibody
TPO	Thyroid peroxidase
TPOAb	Thyroid peroxidase autoantibody
TRAb	TSH receptor autoantibody
TRH	L-pyroglutamyl-L-histidyl-L-proline amide
Tris	Hydroxylmethyl-methylamine
TSAb	Thyroid-stimulating hormone Antibody
TSH	Thyroid-stimulating hormone (thyrotropin)
TSI	Thyroid-stimulating immunoglobulin
U.K.	United Kingdom
UV	Ultraviolet

Abstract

The presence of antibodies to thyroid peroxidase (i.e. TPOAb) in human serum has been measured using an immunoradiometric assay (IRMA), an enzyme-immunometric assay, and three other immunoassay systems which incorporated the chemiluminescent label acridinium ester (AE).

The IRMA successfully utilised a commercial preparation of TPO labelled with [^{125}I] and magnetic particles coupled to Protein A (*Staphylococcus aureus)* (MPA), with the latter separation medium used in an enzyme-immunometric assay which incorporated TPO labelled with horse-radish peroxidase (HRP-TPO). TPO was also labelled with AE and used in a chemiluminometric assay with magnetic particles coupled to human anti-IgG (MAb-Anti-IgG). The latter assay exhibited an improved response, as compared with the non-viable enzyme-immunometric assay, but was inferior to the IRMA lacking the required sensitivity and precision for a viable, clinical assay.

A competitive assay system was also investigated which utilised human anti-TPO (obtained from a purified preparation of IgG) labelled with acridinium ester (Acrid-IgG), TPO labelled with biotin (Biotin-TPO) and magnetic streptavidin-labelled 'Dynabeads®', as the separation medium. This immunoassay produced a similar 'blanket' response as produced in the enzyme-immunometric assay. The coated-tube assay involved the immobilisation of TPO onto plastic tubes and the use of a commercial preparation of sheep anti-human IgG labelled with AE (Acrid-Anti-IgG). This system proved to be the most viable of all the non-radioactive assays, with the results comparing favourably with the established ELISA with a correlation coefficient (r) of 0.96 and P = <0.001, (using the 'least squares linear regression after logarithmic conversion of the data). A good correlation was also demonstrated in the Deming and the Passing & Bablok plots. The coated-tube assay also compared favourably with indirect agglutination (r) = 0.80, P = <0.001.

The results indicated that the less pure source of TPO used in the non-radioactive assays, could only be successfully applied to the measurement of TPOAb, when immobilised onto a solid-phase support such as the ELISA micro-titre plate or onto plastic tubes, but not in the more random, assay systems which utilised magnetic particles as the separation phase. The solid-phase, coated-tube chemiluminometric assay was comparable with ELISA for the measurement of TPOAb in human serum.

CHAPTER 1

INTRODUCTION

Chapter 1: Introduction

1.1 The Thyroid Gland

Disease of the thyroid gland is common and, although not usually life threatening, is quite prevalent in the general population and can be quite debilitating in some circumstances, affecting both the physical and mental well-being of the sufferer. Thyroid disease was indicated as early as the 19th century in the medical literature, as shown by the patient in Figure 1.

Figure 1 A 19th century photograph of a patient with autoimmune Graves' disease. Photograph courtesy of the Royal College of Physicians of Edinburgh [Becton Dickinson (U.K.) Ltd.].

The adult thyroid gland is a highly vascular structure of about 25g in weight, and consists of two conical lobes joined by an isthmus and is located anterolaterally to the trachea, just behind its junction with the larynx (Williams & Goodburn, 1983). The main functions of the gland are concerned with the trapping of iodide (by active transport from the blood); the concentrating (and subsequent conversion to iodine) of iodide ions; the synthesis of thyroid hormones [thyroxine (T_4), tri-iodothyronine (T_3) and reverse tri-iodothyronine

(r-T_3)], (via phenolic coupling) by the follicular cells (Doniach, 1967); their storage in the follicular colloid, and their subsequent release into the bloodstream. The formation of mono-iodotyrosine (MIT) or di-iodotyrosine (DIT), which combine to form the thyroid hormones, T_3 and T_4, occurs via the iodination of thyroglobulin (Tg), catalysed by thyroid peroxidase (TPO) (Williams & Goodburn, 1983) [see Figure 2].

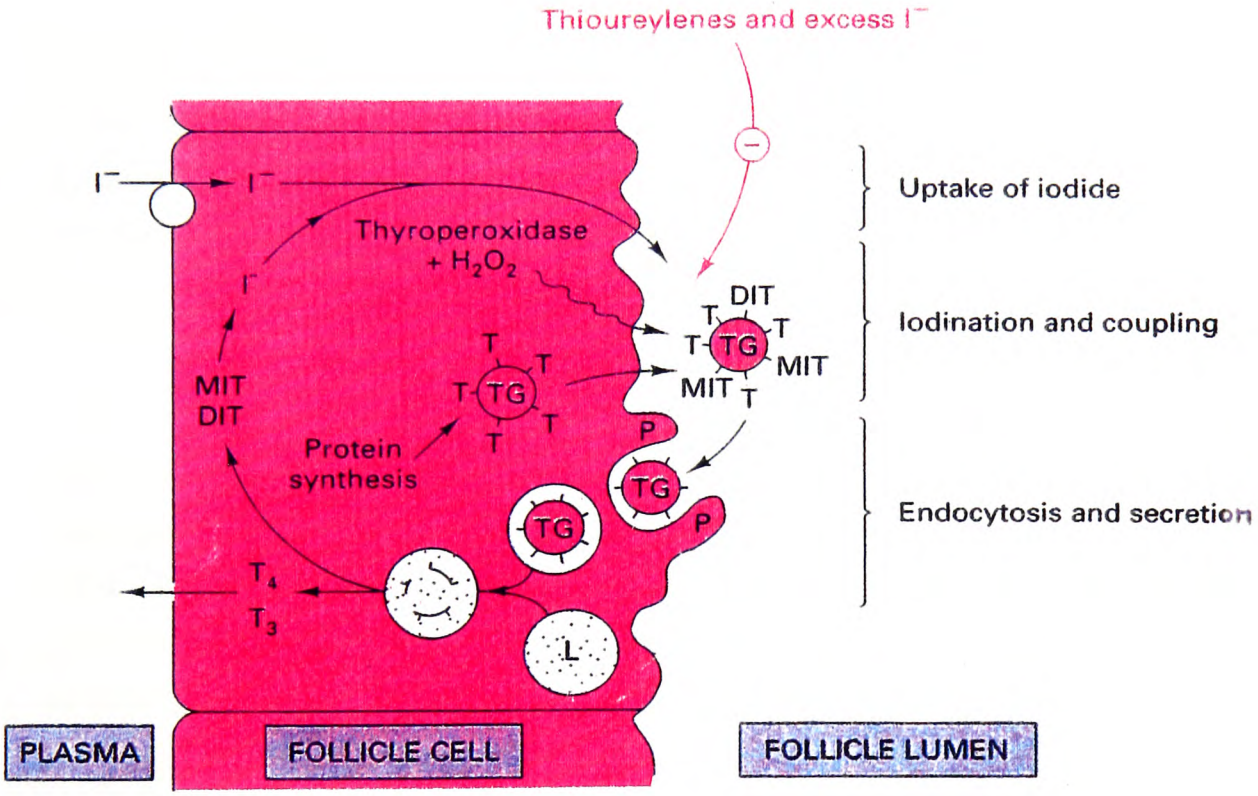


Figure 2 A schematic diagram showing the synthesis of thyroid hormones by the thyroid follicular cell. (Tg = thyroglobulin, T = tyrosine, MIT = monoiodotyrosine, DIT = di-iodotyrosine, T3 = tri-iodothyronine, L = lysosome and p = pseudopod).

[Taken from Pharmacology by Rang, Dale and Ritter, 1995, Churchill Livingstone, New York].

The inorganic iodide absorbed from the diet is actively taken up by the thyroid gland where, in the presence of hydrogen peroxide (H_2O_2) and TPO, it is converted to iodine (Banga *et al.*, 1991). In cell-free systems the peroxidase can be shown to produce molecular iodine (I_2):

$2I^- + H_2O_2 + 2H^+ \text{--------}\rightarrow I_2 + 2H_2O$ (Williams & Goodburn, 1983).

The two-electron oxidation of TPO by H_2O_2 produces an oxoferryl porphyrin pi-cation radical compound I, which is the catalytic species that effects iodide ion oxidation; this compound isomerises spontaneously to produce a protein radical compound, which is thought to catalyse the phenolic coupling reaction of the thyroid hormones (Doerge & Divi, 1995). In the living cell the iodination of thyroglobulin occurs on the surface of the thyroid peroxidase enzyme (a complex, transmembrane protein) located on the apical membrane of the thyrocyte (Banga *et al.*, 1991).

The free thyroid hormones (the biologically active components), are essential for growth and development, and have a central role in cell metabolism and the rate of metabolic processes (Henneman *et al.*, 1994), including effects on heat production, neuromuscular activity and protein synthesis (Williams & Goodburn, 1983). The glycoprotein, thyrotropin (thyroid stimulating hormone, TSH) initiates all pathways involved in the synthesis of the thyroid hormones (Henneman *et al.*, 1994), and together with the tri-peptide thyrotrophin releasing hormone, TRH, (L-pyroglutamyl-L-histidyl-L-proline amide), which is produced in the hypothalamus, is involved in the thyroid negative feedback, hypothalamic-pituitary control mechanism as illustrated in Figure 3 (Williams & Goodburn, 1983).

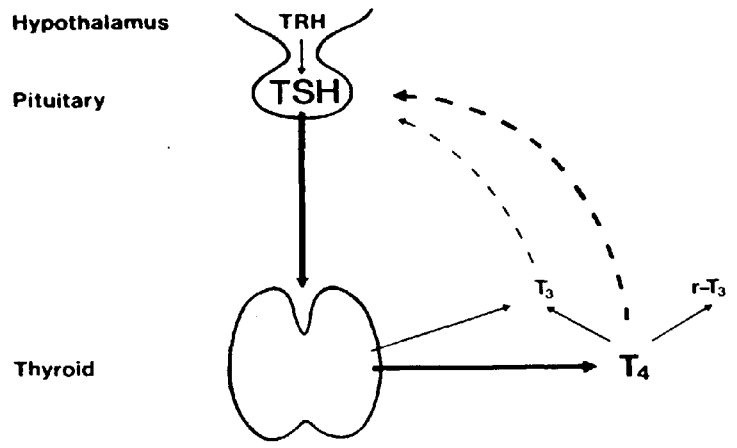


Figure 3 Secretion, metabolism and control of thyroid hormones.
Solid lines - secretion and metabolism of hormones;
Dotted lines - negative feedback mechanisms.

[Taken from 'Clinical Chemistry in Diagnosis and Treatment' by Zilva and Pannall, 1984, 4th Edition, Lloyd-Luke (Medical Books) Ltd., London].

1.2 Autoimmune Thyroid Disease (AITD)

The thyroid gland can be affected by the same spectrum of disease processes that can strike other organs and tissues, including autoimmune disease (Williams & Goodburn, 1983). The aetiology and pathology of thyroid disease is more complex than initially comprehended and increased interest has been focused on the autoimmune variants of disease and its implications. The clinical aspects of autoimmune thyroid disease (AITD) are diverse, with the thyroid gland being much more prone to autoimmune disease than other endocrine glands (Williams & Goodburn, 1983).

The function of the immune system is to identify and eliminate foreign protein antigens from the body, controlled through a process known as tolerance (Roitt *et al.*, 1996; Janeway & Travers, 1996). The breakdown in tolerance and immunoregulation results in autoimmunity, with the generation of humoral and/or cell mediated activity directed against a self protein resulting in tissue destruction (Roitt *et al.*, 1996; Janeway & Travers, 1996).

The prevalence of AITD in the general population is quite high (Banga *et al.*, 1991), affecting approximately 5% of the population (Weetman & McGregor., 1994), and consequently has been intensively studied (Williams & Goodburn, 1983). Although the pathology of these diseases is well-documented, their origins are unclear (Weetman & McGregor, 1994). Autoimmune thyroid disorders include Graves' disease (GD), Hashimoto's thyroiditis (HT), post-partum thyroiditis (PPT), primary myxoedema, and focal lymphocytic thyroiditis.

Individuals with AITD demonstrate an increased incidence of other autoimmune disease, such as systemic lupus erythmatosis (SLE) and Addison's disease (Williams & Goodburn, 1983), and particularly diabetes mellitus (DM), and pernicious anaemia (PA) (Banga *et al.*, 1991), indicating the polyclonal activation of the autoimmune reaction in organ-specific autoimmune diseases (Morita *et al.*, 1995). Paggi *et al.* (1994) showed an increased incidence of anticardiolipin antibodies [associated with systemic lupus erythematosus (SLE)], in patients with AITD. Similarly, Morita *et al.* (1995) showed a high prevalence of anti-nuclear antibody (ANA), anti-smooth muscle antibody (SMA) and anti-ssDNA antibody in some individuals with AITD.

Weetman *et al.* (1983) stated that microsomal autoantibodies (TPOAb) were found in 11.6% of patients with rheumatoid arthritis (RA) and also an increased frequency of thyroid autoantibodies has been reported in Alzheimer patients (Mariotti *et al.*, 1995). This could indicate a possible overlap in the pathogenicity of these conditions (Morita *et al.*, 1995), but may merely represent a non-specific marker of immune disregulation (Mariotti *et al.*, 1995).

Patients with AITD present with a spectrum of clinical disease, from immune destruction as in Hashimoto's thyroiditis (HT), which leads to hypothyroidism, to excessive stimulation which results in Graves' disease (GD) and hyperthyroidism (Banga *et al.*, 1991). GD and HT are both considered to be

organ-specific autoimmune diseases (Morita *et al.*,1995), and are examples of type II autoimmune polyglandular syndrome (APS) (Riley, 1995).

Hypothyroidism presents with non-specific symptoms such as lethargy, mild depression, variation in menstruation and weight increase, together with the classic features which consist of bradycardia, dry skin and hair loss, with the myotonic ankle jerk being the most discriminatory clinical feature (Lazarus, 1996).

GD in contrast, is associated with hyperthyroidism, which includes the symptoms of sweating, palpitations, irritability, insomnia, mood swings, tremor, ocular signs (exophthalmopathy), smooth skin, heat intolerance, nervousness and tachycardia, together with a generalised increase in metabolic rate (Williams & Goodburn, 1983).

1.2.1 Hashimoto's Thyroiditis (HT)

Hashimoto's thyroiditis (HT) was first described in 1912 and is the most common organ-specific autoimmune disease in man (Roitt *et al.*, 1989), and is the most prevalent cause of primary thyroid failure (Lazarus, 1996). HT is a T cell mediated disease (Rieu *et al.*, 1994) and is characterised by infiltration of the gland by lymphocytes, with gradual destruction of the gland (Tomer, 1997). The gland is lobulated (Lazarus, 1996), enlarged and horseshoe-shaped, with a firm, rubbery consistency (Williams & Goodburn, 1983).

The peak incidence occurs between 50 and 60 years of age and the disease is more common in women and Caucasians (Volpé, 1989a). The incidence is thought to have increased in recent years due to a greater intake of dietary iodine (Weetman & McGregor, 1994). The thyroid's functional state may vary through the course of Hashimoto's disease (Rieu *et al.*, 1994). Most characteristically, although patients may remain euthyroid for many years, approximately 10% will ultimately become hypothyroid (Gorin & Lamberg, 1975). Other associated hormonal deficiencies may contribute to the clinical picture (e.g. features of hypogonadism) (Lazarus, 1996).

1.2.2 Graves' Disease (GD)

Graves' disease (GD), also known as Parry's disease, von Basedow's disease, exophthalmic goitre and toxic diffuse goitre, is an autoimmune disease (Weetman & McGregor, 1994; Volpé, 1994), which (as in HT), is characterised histologically by lymphocyte infiltration of the thyroid (Cavan *et al.*, 1994) and is associated with a diffusely enlarged, non-nodular gland (Williams & Goodburn, 1983). GD is again more prevalent in females (4-5 times more), and increases with age (Volpé, 1989b). In HT, animal models have contributed to an improved understanding of the disease, however, an equivalent model is not currently available for GD (Weetman & McGregor, 1994). GD best fits a polygenic, multifactorial model of disease in which genetically susceptible individuals are exposed to a constitutional or environmental insult. This provocation can then result in the activation of the immune response and trigger the B cell production of thyroid-receptor autoantibodies (TRAb), which can lead directly to the induction of hyperthyroid GD (Weetman & McGregor, 1994).

Both GD and HT are characterised by the presence of autoantibodies against the thyroid tissue-specific components thyroid peroxidase (TPO) (i.e. TPOAb) and thyroglobulin (Tg) (i.e. TgAb). GD is also characterised by the presence of TSH receptor autoantibodies (TRAb) (Volpé, 1989b; Banga *et al.*, 1991; Weetman & McGregor, 1994), such as TSH binding inhibitory immunoglobulin (TBII) and thyroid stimulating antibodies (TSAb), [also called thyroid-stimulating immunoglobulin (TSI)] (Miles *et al.*, 1998). The latter was initially discovered by Adams and Purves in 1956 and termed Long Acting Thyroid Stimulator (LATS) (Williams & Goodburn, 1983).

1.2.3 Post-partum Thyroiditis (PPT)

Post-partum thyroiditis (PPT) is a transient, destructive, autoimmune syndrome, similar to Hashimoto's thyroiditis (Parkes *et al.*, 1994). PPT affects 4-7% (Jansson *et al.*, 1988; Gerstein, 1990), 5-9% (Othman *et al.*, 1990), 4-6% (Weetman, 1994) of apparently healthy women in the first year

after delivery. The syndrome is characterised biochemically by an episode of hypothyroidism or sequential episodes of both hyper and hypo-thyroidism (Parkes *et al.*, 1994).

An elevated level of thyroid peroxidase autoantibodies (TPOAb) during early pregnancy is generally considered to be the best predictor of PPT (Jansson *et al.*, 1984; Weetman, 1992; Creagh *et al.*, 1994; Weetman & McGregor, 1994). Symptoms of PPT appear in some 50% of women who have positive TPOAb levels at 16 weeks gestation (Lazurus & Othman, 1991; Creagh *et al.*, 1994; Lazurus *et al.*, 1996). In 20 - 30% of the women who develop PPT, the hypothyroid state becomes permanent (Jansson *et al.*, 1984; Othman *et al.*, 1990; Creagh *et al.*, 1994; Lazurus *et al.*, 1996).

An association between depression and post-partum thyroiditis certainly exists in the hypothyroid state, but severe psychiatric symptoms are uncommon (Harris *et al.*, 1992; Pop *et al.*, 1995). PPT has been associated with decreased foetal size and lower birth weight (Weetman & McGregor, 1994) and later impaired child development (Pop *et al.*, 1995), and also with the devastating consequences of increased infant mortality rate and spontaneous abortion (Burrow, 1993; Pop *et al.*, 1995).

1.3 Factors Implicated in Autoimmune Thyroid Disease (AITD)

Genetic predisposition, together with ill-defined environmental and endogenous factors, determines susceptibility to AITD (Weetman & McGregor, 1994). AITD clearly increases with age (especially) in the female population (Fong *et al.*, 1981; Roti *et al.*, 1992; Weetman, 1992; Mariotti *et al.*, 1995; Sunbeck *et al.*, 1995). The hormone oestrogen has been associated with the exacerbation of AITD, in contrast to testosterone which has been associated with the amelioration of AITD (Weetman, 1991). Prolactin may also have a role because asymptomatic autoimmune thyroiditis is more common in hyperprolactinaemic disorders (Ferrari *et al.*, 1983). AITD is also known to present during puberty, pregnancy, postpartum period

(Weetman, 1991) and the menopause (Williams & Goodburn, 1983; Vanderpump *et al.*, 1995).

GD is diagnosed more frequently in the warmer months of the year, which could possibly be due to the increased heat-intolerance experienced (Weetman & McGregor, 1994; Westphal, 1994). Seasonal variation could also be attributed to an associated increase in iodine intake, which has also been implicated in the onset and severity of GD (Weetman & McGregor, 1994), and the enhancement of thyroid autoimmunity in genetically susceptible individuals (Prentice *et al.*, 1990). Sudden changes in iodine intake may be more important for the development of thyroiditis and thyroid autoantibodies than prolonged exposure to a high but constant iodine intake (Weetman & McGregor, 1994).

Stress has been associated with the onset of thyroid disease (Kanner *et al.*, 1981; Leclere & Weryha, 1989; Weetman & McGregor, 1994; Kung, 1995). Recent studies have demonstrated a shared antigenicity of the human stress protein (hsp70) with thyroid peroxidase (Youde & Parkes, 1996a).

The administration of various cytokines [i.e. alpha-interferon (α-IFN)] is a clearly established iatrogenic cause for AITD (Weetman & McGregor, 1994). Clinical studies have suggested that lithium may dispose individuals to the development of AITD (Calabrese *et al.*, 1985; Lazurus *et al.*, 1986) and Graves' disease can develop in patients on long-term therapy (Barclay *et al.*, 1994). However, studies by Lee *et al.* (1992) on a Hong Kong population in contrast, indicated that thyroid autoantibodies were not as prevalent in their lithium patients, possibly attributable to ethnic (genetic) differences, and indicated the multifactorial mechanisms of lithium-associated thyroid abnormalities. Cyclosporin (CS) a potent immunosuppressive agent has been shown to aggravate and/or induce relapse in AITD (Prud'homme *et al.*, 1991).

Infection with microorganisms has been linked to the initiation of autoimmune thyroid disease, although definitive roles for their involvement have not been proven (Weetman & McGregor, 1994). These include *Yersinia enterocolitica*

associated with food poisoning (Weiss *et al.*, 1983); congenital rubella syndrome linked with thyroid disease (Clarke *et al.*, 1984); hepatitis C and Graves' disease (Leri *et al.*, 1992); influenza, Coxsackie, adenovirus and mumps (Weetman & Borysiewicz, 1990); and the Epstein-Barr virus (EBV) (Coyle *et al.*, 1989).

The major histocompatibility complex (MHC) of genes, called HLA (Human Leucocyte Antigen) in man, controls (in part) immune responsiveness to a variety of foreign and self antigens (Weetman, 1992). Individuals with certain HLA profiles seem prone to various forms of thyroid disease (Cavan *et al.*, 1994; Nicholson *et al.*, 1994). GD is associated with particular HLA types, but the associations are relatively weak and inconsistent between populations (Weetman, 1992), and it seems that the HLA genes may be important in conferring protection against GD (Cavan *et al.*, 1994). The available data suggest strongly that a non-MHC genetic factor plays an important role in susceptibility (Weetman & McGregor, 1994). Thyroid autoimmunity has been associated with familial Alzheimer's disease and Down's syndrome, suggesting the influence of an unknown genetic factor on chromosome 21 (Ewins *et al.*, 1991). Thyroid abnormalities in Down's syndrome however, appear to be heterogeneous in origin and are not always associated with autoimmunity (Ewins *et al.*, 1991). AITD has also been associated with Turner's syndrome (Williams & Goodburn, 1983).

1.4 The Major Autoantigens

Thyroid peroxidase (TPO), formerly known as the microsomal antigen, together with thyroglobulin (Tg) and the TSH-receptor (TSH-R) are the three major autoantigens involved in AITD. Undoubtedly, the major advance in thyroid autoimmunity in the last decade was the cloning and sequencing of the three thyroid autoantigens, contributing to a detailed characterisation of the autoantibodies (Weetman & McGregor., 1994). All of these autoantigens play essential roles in thyroid hormone synthesis [see Figure 4 for diagrammatic representation of the major thyroid autoantigens].

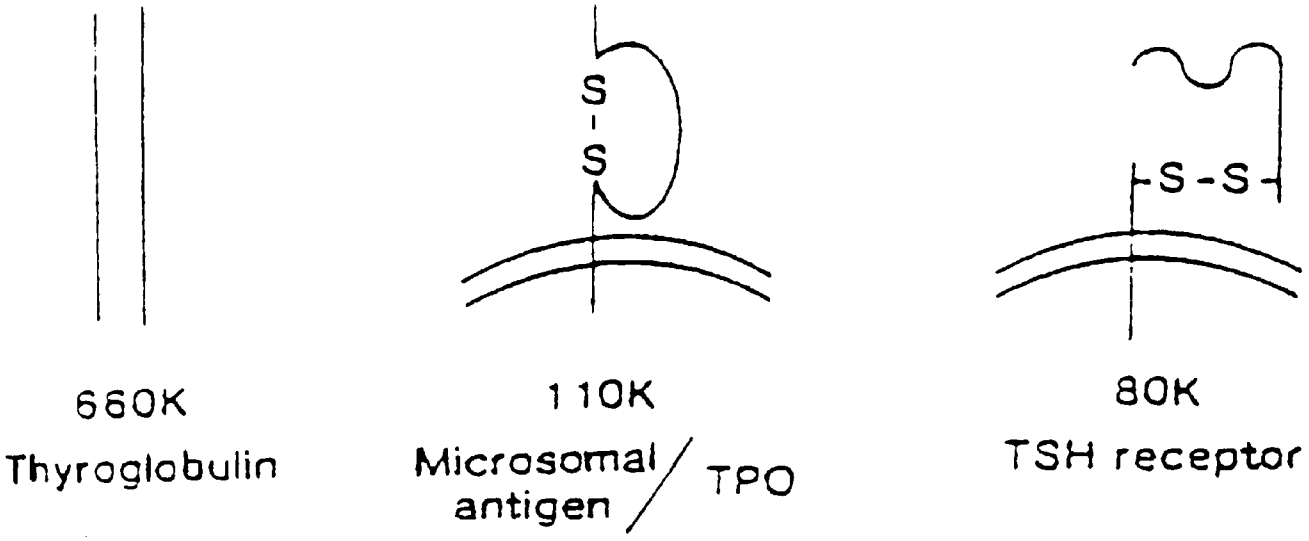


Figure 4 A schematic diagram showing the structures of the three major autoantigens involved in Graves' disease and Hashimoto thyroiditis [O'Connor & Davies, 1990].

1.4.1 Thyroid Peroxidase (TPO)

Thyroid peroxidase (TPO) has been described as a small membrane-bound glycoprotein of 100-105 kilodaltons (kDa), which contained a haem group with a central role in tyrosyl residue iodination and coupling (Banga *et al.*, 1984; Weetman & McGregor, 1994). Subsequent immunoprecipitation and Western blotting studies confirmed the molecular weight of TPO to be 103-110 kDa under reducing conditions and from 117 kDa under non-reducing conditions (Banga *et al.*, 1991). However, McLachlan & Rapoport, 1995, claimed that TPO is a membrane-bound glycoprotein comprising two identical approximately 100 kDa subunits. The 93 kDa extracellular region of TPO can be purified in limited amounts from thyroid tissue and it is this region which is particularly suitable for the studies of the associated human TPO autoantibodies (McLachlan & Rapoport, 1995). X-Ray crystallography

has shown that TPO is composed mainly of alpha-helical structure with little beta-sheet structure (Banga *et al.*, 1991).

The antigen was first recognised by immunofluorescent studies some 40 years ago by Trotter *et al.* (1957). The antigen was located to the microsomal fraction of the thyroid gland (Roitt & Doniach, 1958) and later linked to the smooth-surface vesicles of the microsomal fraction of the thyroid gland (Belyavin & Trotter, 1959; Roitt *et al.*, 1964). Khoury *et al.* (1984) later provided evidence that in AITD, microsomal autoantibodies were present in the apical portion of follicular cells, the probable site of TPO-catalysed iodination, which was similarly demonstrated by Aliquer *et al.* (1989).

The nature of this antigen remained unknown for more than 20 years after its discovery, but it become clear that the thyroid microsomal antigen was very closely related to, if not identical with thyroid peroxidase (TPO) (Doniach *et al.*, 1982; Portmann *et al.*, 1985; Ruf *et al.*, 1985; Kotani *et al.*, 1986; Nakajima *et al.*, 1987; Yokoyama *et al.*, 1989; Roti *et al.*, 1992). It has now been shown to be identical to thyroid peroxidase (TPO) (Aliquer *et al.*, 1989; Banga *et al.*, 1991). The coding sequence for the human thyroid peroxidase (hTPO) complementary DNA (cDNA) comprises 2799 nucleotides and codes for a 933 amino acid protein with five potential N-glycosylation sites. A typical hydrophobic sequence of 25 residues is found towards the C-terminus of the molecule. This traverses the plasma membrane and anchors the enzyme to the apical surface of the thyroid follicular cell (Banga *et al.*, 1991). Two forms of TPO referred to as TPO-1 (larger) and TPO-2 (smaller) have been described (Banga *et al.*, 1991).

The autoimmune response to TPO is heterogeneous and polyclonal in nature (Banga *et al.*, 1991). Epitopes of TPO have been found to be both conformational and linear (Ludgate *et al.*, 1989; Finke *et al.*, 1990). Kohno *et al.* (1986) concluded that at least three epitopes of TPO were recognised by serum autoantibodies in their study, but up to six autoantigenic epitopes have been recognised by others (Hamada *et al.*, 1987; Weetman *et al.*, 1987; Doble *et al.*, 1988; Banga *et al.*, 1991). Ruf *et al.* (1989) mapped four antigenic domains (A,B,C and D) on the TPO molecule, with seven epitopes

(recognised by monoclonal antibodies), localised in the four antigenic domains. The variable outcome in results can probably be attributed to the different investigative techniques employed.

One particular epitope, an 85 amino acid segment of TPO, is known as C2 (Ludgate *et al.*, 1989). This sequence harbours a major epitope recognised in the serum of patients with both GD and HT. It has been suggested that TPO and Tg may share common epitopes (Kohno *et al.*, 1988; Banga *et al.*, 1991), and Weetman & McGregor (1994) state that there is some conformational similarity between TPO and Tg. If this proves to be the case, it will unveil novel explanations for the simultaneous presence of high levels of Tg and TPO autoantibodies in the majority of AITD patients (Banga *et al.*, 1991). Weetman & McGregor (1984) and Ruf *et al.* (1993) also reported the identification of a new category of thyroid-specific autoantibody interacting with both Tg and TPO (TGPO autoantibodies).

1.5 Thyroid Peroxidase Autoantibodies (TPOAb)

Thyroid peroxidase autoantibodies (TPOAb), [formerly termed microsomal autoantibodies (MicAb)], are considered to be a more specific indicator of AITD than thyroglobulin autoantibodies (TgAb). Early studies demonstrated the presence of TPOAb in virtually all cases of HT (Trotter *et al.*, 1957; Belyavin & Trotter, 1959), while only some 70% of patients had TgAb (Roitt *et al.*, 1956). More recently, TgAb have been found in no more than 50% of lymphocytic thyroiditis patients and 25% of GD patients, whereas TPOAb are found in over 90% of both groups of patients (Banga *et al.*, 1991). A positive correlation between TPOAb and the intensity of the intrathyroidal autoimmune process in GD has been described (Paschke *et al.*, 1993). However, Mariotti *et al.* (1990) found no simple relationship between TPOAb and thyroid status in their study.

The TPOAb was discovered in the serum of patients with HT due to differences between the precipitation and complement-fixing reactions of

TPOAb as compared with TgAb. The TPOAb were capable of complement fixation, unlike TgAb (Belyavin & Trotter, 1959).

The TPOAb have an IgG subclass distribution similar to those against Tg (Parkes *et al.*, 1984; Banga *et al.*, 1991; Weetman & McGregor, 1994; McLachlan & Rapoport, 1995). Preliminary studies indicated a predominance of subclasses IgG1 and IgG4 in patients' sera (Parkes *et al.*, 1984). However, in a review by Banga *et al.*, 1991, they state that subsequent studies by Weetman *et al.* (1989) demonstrated the significant presence of IgG2 subclass TPOAb, with a higher affinity for TPO than IgG1 and IgG4. This could therefore represent the biologically important subclass in this disease (Banga *et al.*, 1991). Guo *et al.* (1997) also provided evidence for the presence of TPOAb of IgE class in AITD.

The thyroid receptor autoantibodies (TRAb) are directly pathogenic, whereas the involvement of TgAb and TPOAb in the pathogenesis of the disease has been unclear, and they have been considered to be innocent bystanders, reflecting the cellular destruction of the underlying gland, and used as diagnostic markers (Riley, 1995). However, there is growing evidence to suggest that disease-associated TgAb may play a role in the initiation of autoimmune thyroiditis (Tomer, 1997). The TPOAb are complement fixing and this raises the possibility that they may also play a role in the pathogenesis of thyroid follicular cell destruction in lymphocytic thyroiditis (Banga *et al.*, 1991). Preliminary studies by Parkes *et al.* (1993) indicated that thyroid damage during the post-partum period may be mediated via the complement system.

1.6 Prevalence of Thyroid Peroxidase Autoantibodies in the Normal Population

It is generally agreed that circulating thyroid autoantibodies are the hallmark of AITD (Yoshida *et al.*, 1978; Weetman & McGregor 1984; Mariotti *et al.*, 1990; Fan *et al.*, 1994; McLachlan & Rapoport, 1995; Sundbeck *et al.*, 1995), but low levels of TPOAb [and TgAb (Tomer, 1997)] are detected in

apparently normal individuals, which could indicate a predisposition to the development of disease. It is not clear which individuals will be affected by disease (Hidaka *et al.*, 1994), but a recent follow-up study indicated a continuing risk of developing thyroid failure in euthyroid persons, with positive thyroid autoantibodies with a female/male ratio of about 5 to 1 (Vanderpump *et al.*, 1995).

A study by Yoshida *et al.* (1978) indicated a very high prevalence of individuals with asymptomatic autoimmune thyroid diseases in the normal population. However, widely differing figures have been quoted, varying from 3.6-8.4% of apparently normal adults (Roti *et al.*, 1992; Sundbeck *et al.*, 1995), to 7% (Tunbridge *et al.*,1977), 8.4% (Mariotti *et al.*, 1990), 10% (Groves *et al.*, 1990), and 15% (Weetman & McGregor 1992). The observed increase in percentage values in more recent studies is probably a reflection of the improved sensitivity of assays.

The age-dependent increase in the prevalence of positive TgAb and TPOAb is a well established phenomenon, recognised since the early 1960s (Hackett & Beech 1960; Hijmans *et al.*, 1984), with TPOAb having been detected in 8% of children (Roti *et al.*, 1992). However, some reports indicate decreasing prevalence of TPOAb at ages greater than 60 years, thus indicating the need for a better understanding of the diagnostic and prognostic significance of increased TPOAb concentrations in the elderly (Sundbeck *et al.*, 1995).

There is an increased prevalence of TPOAb in the female population with Roti *et al.* (1992), quoting values of 2.3% in elderly men as opposed to 10.2% in elderly women. In the Wickham study in North-East England, MicAb were detectable in 7.9% of young women (aged 18-24) rising to 13.7% at age 45-54 years (Tunbridge *et al.*, 1977), with values of 14.9% and 24.2% quoted for the age-groups (18-24) and (55-64) years respectively, in women blood-donors by Prentice *et al.* (1990).

Different reports of the prevalence of autoantibodies in both diseased and apparently normal individuals may be attributable to the use of different study designs, decision limits and subject populations (i.e. variation due to regional

differences, including ethnic background, iodine intake, gender ratio and age). In addition, TPOAb are heterogeneous so that different methods (i.e. of sensitivity and specificity) may have given different results in different thyroid disorders (Sundbeck *et al.*, 1995).

1.7 Methods for Measuring Thyroid Autoantibodies

Autoantibodies present in biological fluids are identified exclusively by highly specific and sensitive immunochemical techniques, which involve the detection of a complex formed between the antibody and a target antigen (using antibodies or antigens labelled with various markers), which is presented in tissues, cells, a crude tissue extract, or in a purified form (Miles *et al.*, 1998). Methods for measuring MicAb/TPOAb, TgAb, and TRAb, have improved over the last decade. However, more accurate and sensitive methods, together with high sample throughput are increasingly needed for identifying patients with autoimmune thyroid disease (AITD) and individuals at high risk for the possible onset of thyroid autoimmunity e.g. pregnant and postpartum females [especially in females with Type 1 Diabetes Mellitus (Bech *et al.*, 1991)], and individuals with a family history of these diseases.

In most cases TPOAb measurement is sufficient, and it is generally agreed that TPOAb appears to be a more sensitive indicator of AITD than TgAb (Banga *et al.*, 1991) and the more clinically relevant (Miles *et al.*, 1998). The measurement of TgAb however, is pertinent for disclosing possible interference in the methods for measuring Tg as a tumour marker in thyroid carcinoma (Feldt-Rasmussen, 1996). Many investigators have mentioned the usefulness of TRAb measurement in the diagnosis of GD (Kasagi *et al.*, 1987; Chiovato *et al.*, 1994; Mukata *et al.*, 1994) and as an indicator of individuals at high risk of developing PPT (Hidaka *et al.*, 1994). However, as TRAb methodologies have been technically complex and relatively insensitive, with no recommended methods, accepted standards or quality assurance schemes for their measurement they have not been widely adopted for routine use (Feldt-Rasmussen, 1996; Miles *et al.*, 1998).

1.8 Methods for the Detection and Measurement of Microsomal/Thyroid Peroxidase Autoantibodies (MicAb/TPOAb)

New molecular and biochemical techniques, together with the improvement in separation procedures have all contributed to more precise antigen identification and to the availability of highly purified antigen preparations of thyroglobulin (Tg) and thyroid microsomal antigen [thyroid peroxidase (TPO)] from the thyroid gland (Miles *et al.*, 1998). In earlier methodologies such as indirect immunofluorescence (IMF) and passive haemagglutination (PH), the crude microsomal antigen preparation was used (usually contaminated with Tg) (Feldt-Rasmussen, 1996) and the autoantibodies detected were initially termed microsomal autoantibodies (MicAb). Later assays utilised more purified preparations (including monoclonal antibodies to TPO and recombinant TPO preparations) and were termed thyroid peroxidase autoantibodies (TPOAb).

Thyroid MicAb/TPOAb (and TgAb) have both been traditionally detected or measured using the same methodologies. The assay of thyroid peroxidase autoantibodies by various methods has been used in the diagnosis of AITD since they were first described by Roitt *et al.* (1956). The earlier methods provided a means of detection of the autoantibody only i.e. immunoprecipitation, complement fixation (CF) and indirect imunofluorescence (IMF) (the latter a histological technique used as an autoantibody screen). The semi-quantitative particle agglutination technique was then devised, which was mainly superseded by quantitative procedures such as Radioimmunoassay (RIA), Immunoradiometric assay (IRMA), Enzyme-linked immunosorbent assay (ELISA) and more recently Chemiluminescent immunoassay (CIA). The earlier methods will only be mentioned briefly, with greater emphasis placed on the methods used in this study.

1.8.1 Immunoprecipitation

In immunochemical reactions, addition of an increasing amount of antigen to a constant amount of antibody will result in increasing precipitation which reaches a plateau and then diminishes (Heidelberger & Kendall, 1935). The fundamental aspect of immunoprecipitation provided the basis for the quantitative immunoassay method called the 'precipitin' test, described by Heidelberger and Kendall in 1929 and 1935 (Edwards, 1985), and was used by Roitt *et al.* (1956) for detecting thyroid autoantibodies in Hashimoto's thyroiditis (Anderson *et al.*, 1957), and in 'primary' myxoedema and sub-acute thyroiditis (Doniach & Roitt, 1957). The formed immunoprecipitate was removed by centrifugation, and the protein content of the precipitate assayed by optical density or nitrogen determination (micro-Kjeldahl). The decrease in precipitation at higher antigen concentrations due to the formation of smaller, more soluble complexes, gave rise to the 'prozone' effect due to antigen excess, which produced false negative results (Miles *et al.*, 1998).

Various immunoprecipitation procedures involving diffusion or electrophoretic migration were subsequently developed, using gels as the reaction medium [i.e. single radial immunodiffusion (SRID), and double immunodiffusion]. Immunodiffusion was used for the routine estimation of larger proteins such as the immunoglobulins and in the detection of TgAb. However, the technique was non-quantitative, lacked sensitivity, with a slow end-point development and was prone to give rise to spurious results due to haemolysis, lipaemia and bacterial contamination (Williams & Goodburn, 1983; Edwards, 1985). Techniques based on electrophoretic separation and visualisation (i.e. immunoblotting) were more rapid and are extensively used in current research procedures.

The concentration of formed immunocomplexes can be measured using light scattering principles (i.e. nephelometry or turbidimetry) (Edwards, 1985). This principle was exploited by Harchhali *et al.*, 1994, who applied microparticle-enhanced nephelometry to the detection of human thyroid peroxidase autoantibodies in AITD (Harchhali *et al.*, 1994).

1.8.2 Complement Fixation (CF)

Red blood cells have been used as labels for monitoring and quantifying the immune reaction since the beginning of this century, with early descriptions of the complement fixation process in 1901 (Edwards, 1985). Complement factors bind to some immunoglobulins when aggregated or when antibodies are combined with their specific antigens (Edwards, 1985). Trotter *et al.* (1957), Anderson *et al.* (1957), Roitt & Doniach (1958) and Belyavin & Trotter (1959) demonstrated the ability of MicAb (TPOAb) to fix complement, with these findings confirmed by Irvine (1960), who demonstrated that serum from patients with Hashimoto's disease was cytotoxic for thyroid cells *in vitro* in the presence of complement (Cayzer, *et al.*, 1978). It was also found by Forbes *et al.* (1962), and subsequently by Khoury *et al.* (1981) that exposure of primary cultures of thyroid cells *in vitro* to TPOAb in the presence of complement led to lysis of the cells (Banga *et al.*, 1991).

The early complement fixation methods used for the detection of thyroid autoantibodies (as described by Anderson *et al.*, 1957; Anderson *et al.*, 1959; Belyavin & Trotter, 1959), involved the incubation for 1 hour at 37°C, of suitable dilutions of (heat-inactivated) serum with complement extracted from guinea pig serum. The complement used had been titrated in the presence of antigen against the sensitised haemolytic system, in order to determine the dilution at which 50% red blood cell (RBC) haemolysis occurred [termed 1 minimum haemolytic dose (1MHD)] (Parkes *et al.*, 1991). The antigen (saline extracts of human thyroid) was also added, with the optimal dilution usually found by 'chessboard' titration with dilutions of positive autoantibody sera of known titre, as described by Belyavin, (1953) (Roitt & Doniach, 1958). This was carried out to identify the highest autoantibody titre which did not exhibit the 'prozone' effect (Parkes *et al.*, 1991).

The residual (unreacted) complement was then detected with the addition of a suitable haemolytic system [e.g. a 3% suspension of sheep red cells sensitised with immune horse serum (Anderson *et al.*, 1959)]. The degree of lysis (activated via the complement cascade system) was then observed

after a further incubation period [see Figure 5 for the schematic representation of method principle].

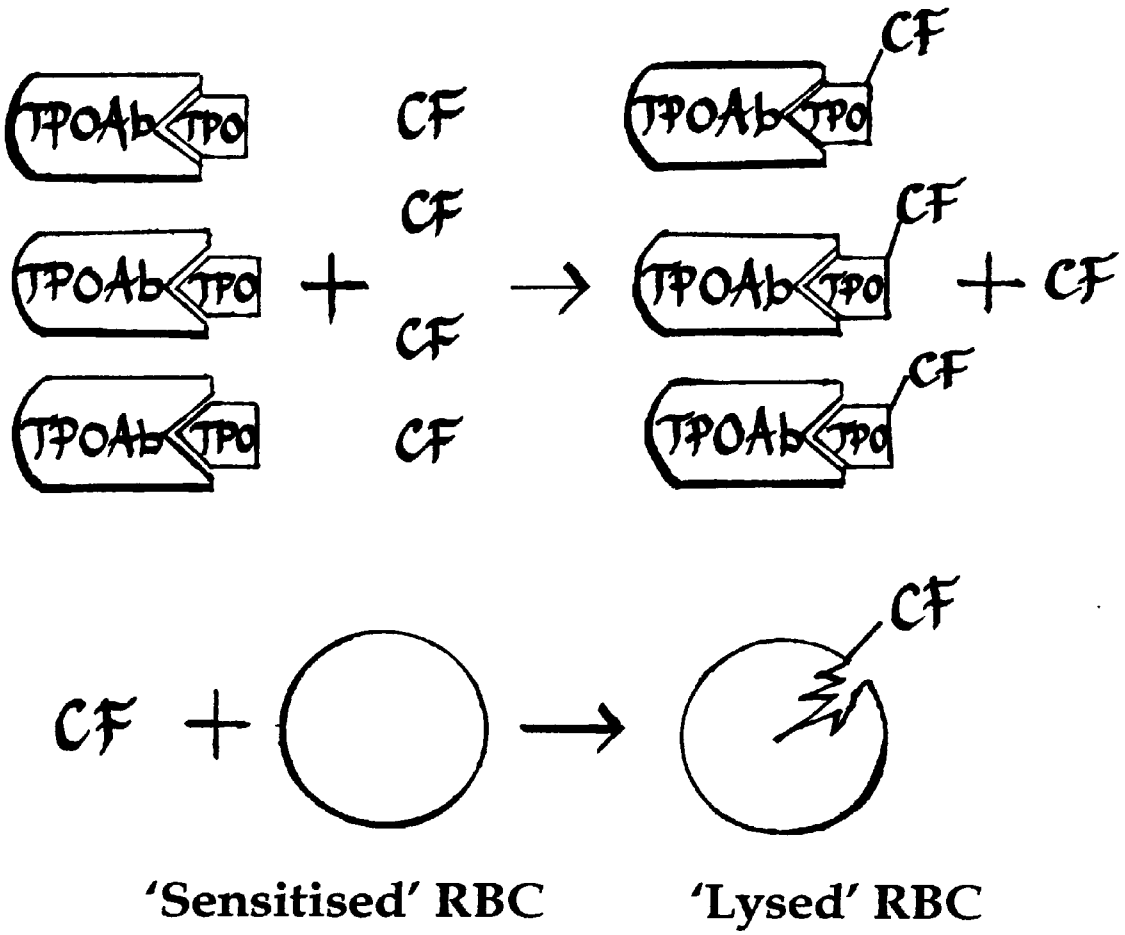


Figure 5 The TPO-TPOAb complex fixes serum complement (CF). Residual complement then causes the lysis of the sensitised red blood cells (RBCs).

Earlier methods could not discriminate between the species of antibody which interacted with the complement system, however, Parkes *et al.* (1991) devised a semi-quantitative micro-method for the differentiation of microsomal (TPO) autoantibodies depending on their degree of complement fixation. The method involved the use of sheep erythrocytes ('sensitised' with rabbit anti-sheep RBC serum) for the detection and titration of complement fixation by autoantibodies directed against human thyroid membranes in the serum of patients with AITD (Parkes *et al.*, 1991).

1.8.3 Indirect Immunofluorescence (IMF)

The secondary thyroid autoantibodies, MicAb/TPOAb and TgAb, were both identified by reaction against tissues or tissue organelles, most often subjectively using indirect immunofluorescence (IMF) microscopy, a relatively simple and inexpensive technique (Miles *et al.*,1998). A composite block of rodent tissues included liver, stomach, kidney and thyroid was used for the so-called 'autoimmune screen' to detect a number of both organ-specific and non-organ specific autoantibodies (Miles *et al.*,1998).

Each antibody was associated with distinctive patterns of fluorescence on human thyroid tissue and the use of a composite tissue block was necessary as anti-mitochondrial antibody demonstrated a similar immunofluorescent staining pattern on thyroid tissue, but could be distinguished from thyroid antibodies by reaction with the other tissues, e.g. liver (Miles *et al.*,1998). Interference from heterophilic antibodies was also evident, with this remaining a pertinent factor in current, sophisticated immunoassays.

Although the immunofluorescence technique was highly sensitive and specific, trained personnel were required to prepare frozen tissue sections [which required a suitable freezing microtome (cryostat)], and also be competent in immunofluorescence microscopy (Ng *et al.*, 1987). Furthermore, being time-consuming and non-quantitative, with the subjective reading of results, it was not amenable to automation and consequently, unsuitable for large-scale screening. As a result immunofluorescence techniques were largely superseded by agglutination [see Figure 6 for the diagrammatic representation of IMF].

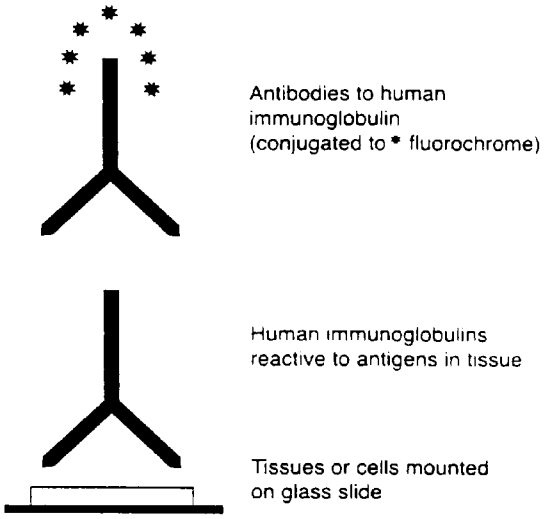


Figure 6 Principle of indirect immunofluorescence.

Human serum under investigation is incubated with tissue mounted on a glass slide. Unbound material is washed from the slide leaving only immunoglobulin specifically recognising antigens in the tissue. Bound human immunoglobulin is detected with an antiserum [i.e. anti-IgG as TgAb and TPOAb are mainly of IgG class (Weetman & McGregor, 1994)] conjugated to a fluorophore [e.g. fluorescein isothiocyanate-conjugate (FITC)] and visualised by fluorescence microscopy (Miles *et al.*, 1998).

1.8.4 Agglutination

Agglutination assays involve the cross-linking of specific antigen-coated particles by an antibody directed against that antigen, to form large macroscopic aggregates (Miles *et al.*, 1998). Boyden (1951) discovered that red blood cells pretreated with tannic acid ('tanned' red cells), passively absorbed exogenous antigens. After absorption of antigen by the tanned red cell, haemagglutination of these cells was a measure of antibodies specific to the absorbed antigen (Edwards, 1985). Bird & Stephenson (1973), Amino *et al.* (1976) & Cayzer *et al.* (1978) described agglutination assays for MicAb using 'tanned' red blood cells as the antigen carrier (coated with human

thyroid microsomal proteins). This technique was termed indirect or passive haemagglutination (PH).

Sheep erythrocytes, initially used, were replaced by avian (turkey) cells as the carrier particle, as they demonstrated advantages of increased specificity (by reducing non-specific agglutination), which varied from species to species (Cayzer *et al.*, 1978). Problems encountered with leaching of antigen from the surface of tanned red cells and non-specific adsorption were overcome with the use of milder and more efficient covalent coupling procedures (i.e. using chromic chloride and glutaraldehyde) (Edwards, 1985). The fragility of prepared red blood cells also resulted in poor storage capacity, with a relatively short shelf-life of 2-3 weeks (Edwards, 1985). However, the stability was increased up to six months at 4°C with the use of formaldehyde-preserved turkey erythrocytes (Cayzer *et al.*, 1978).

Testing was done in the U-shaped wells of agglutination plates so that the pattern could be easily visualised in the bottom of the well. Serial dilutions of samples were made to eliminate problems associated with antigen excess exemplified by the 'prozone' effect encountered in immunoprecipitation techniques. The titre of the antibody was given by the subjective estimation of the highest dilution demonstrating agglutination (Miles *et al.*, 1998).

Haemagglutination assays demonstrated a high degree of non-specific binding on turbid or microbially contaminated sera (Cayzer *et al.*, 1978). High titres of thyroglobulin autoantibodies were also prone to giving false-positive results, which were overcome by adding free thyroglobulin to the microsomal (TPO) diluent (Cayzer *et al.*, 1978; Mariotti *et al.*, 1990). In addition, low titre cross-reacting IgM antibodies, or classic IgM rheumatoid factors, were a potential source of interference (Miles *et al.*, 1998).

Antigen-sensitised turkey thyrocytes, coloured gelatin particles [used in the Serodia®-AMC kit for this project, see Appendix 2:8] or latex were adopted in commercial assays, with the latter options providing more inert, stable systems than the earlier haemagglutination methods. In these assays uncoated particles or cells were used as an internal control to identify

interference from heterophilic antibodies (Miles *et al.*, 1998). However, although agglutination techniques were simple, semi-quantitative and could be used without any sophisticated instrumentation, they were time-consuming, laborious and they lacked the sensitivity of later immunoassays (Mariotti *et al.*, 1990). The subjective evaluation of results (prone to variability in scoring titres) also required experienced interpretative skills as in IMF (Miles *et al.*, 1998). Consequently, agglutination techniques were largely superseded by RIA and ELISA.

1.8.5 Radioimmunoassay (RIA)

The potential advantages of radioimmunoassay (RIA) were sensitivity, specificity, simplicity and a universal application (Edwards, 1985). The background measurement for the radiosotope [^{125}I] in almost all biological samples is virtually zero, giving high sensitivity, and radioactive disintegration is unaffected by changes in physico-chemical parameters such as pH, concentration, or temperature, therefore providing high specificity (Edwards, 1985). The purity of the label needed to be high to enhance the sensitivity and precision of the immunoassay and this was achieved using chromatographic techniques, which produced pure and stable radiolabelled antigens/antibodies (Edwards, 1985). Analagous labelling and purification procedures were used for antigen and antibody, (i.e. using the Chloramine T, Lactoperoxidase, Iodogen and Conjugation labelling procedures and subsequently purified using molecular exclusion chromatograpy) (Edwards, 1985).

The classical, competitive radioimmunoassay (RIA) was based on the reaction of a limited concentration of antibody (reagent) with varying concentrations of antigen and radiolabelled antigen (Edwards, 1985). The converse of this being evident when measuring MicAb/TPOAb i.e. a labelled antibody species would compete with the sample autoantibody for a 'limited' amount of microsomal/TPO antigen [usually immobilised on a plastic support (Mariotti *et al.*, 1987)]. The reaction is allowed to come to a state of

equilibrium, resulting in a mixture of free and bound antibody. The final radioactivity detected in the 'bound' fraction is inversely proportional to the concentration of analyte i.e. MicAb/TPOAb being measured [see Figure 7].

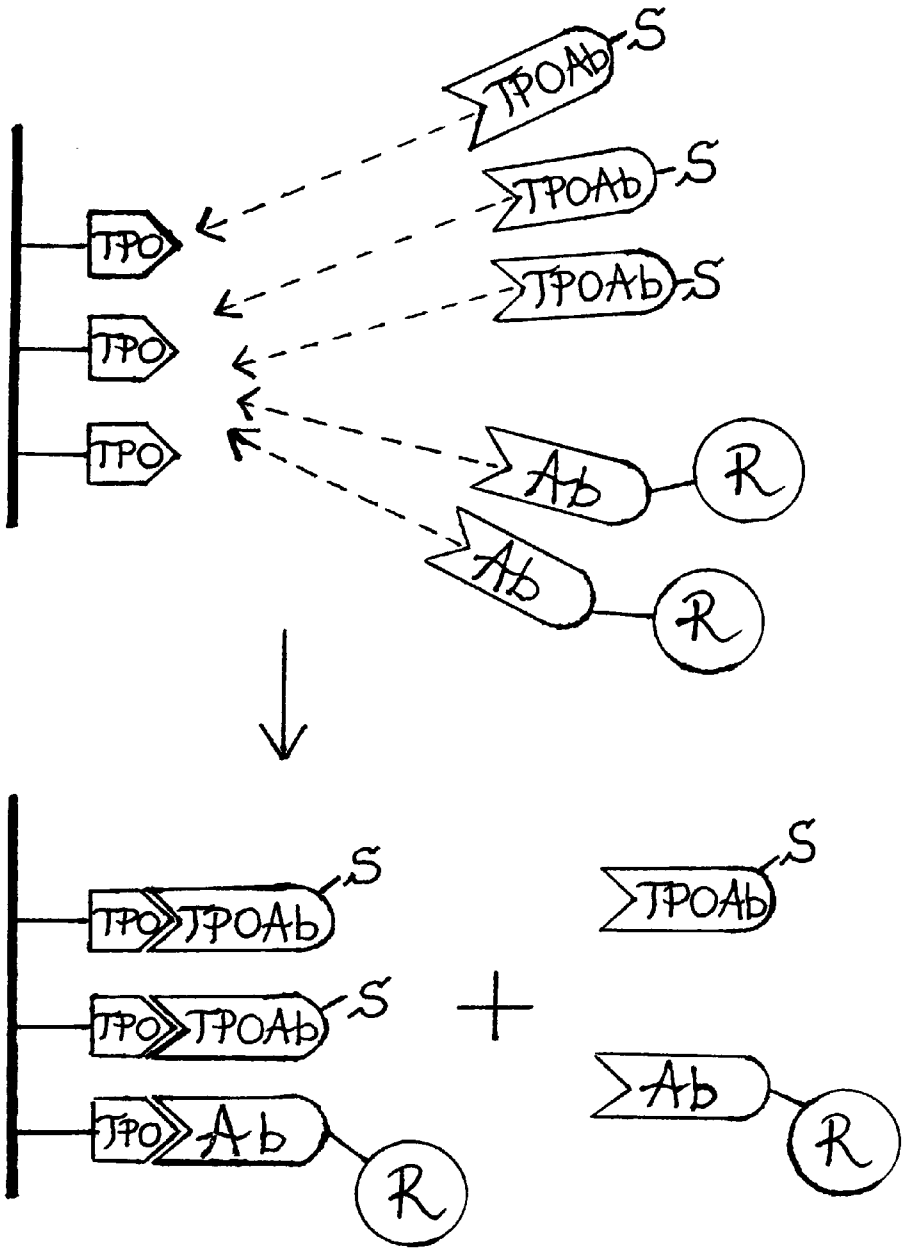


Figure 7 **Radioimmunoassay for measuring serum TPOAb.**
The sample (TPOAb-S) competes with a radioactively-labelled antibody (Ab-R) for the TPO antigen, usually immobilised on a suitable support. The radioactive counts attached to the support via TPO are indirectly proportional to sample TPOAb.

A potentially limiting factor in most radioimmunoassays was the separation of the bound and the free fractions and their subsequent discrimination, which determined the specificity of the assay (Edwards, 1985). This was overcome with the use of pre-formed materials such as tubes and microplates, which greatly improved washing and separation procedures and obviated the need for centrifugation (Voller *et al.*, 1979).

Radioimmunoassays for MicAb/TPOAb required antigenic preparations of sufficiently high purity. In contrast to Tg, TPO being a membrane protein, could only be obtained free of contamination in small quantity (Ruf *et al.*, 1988). This conferred the drawbacks of low specific signal and high non-specific interference from serum components such as Tg with the less pure microsomal preparations (Ruf *et al.*, 1988). This was exemplified in the RIA described by Mariotti *et al.* (1987), to measure the microsomal autoantibody (MicAb) which involved the competition of sample microsomal autoantibody and [^{125}I]-anti-microsomal IgG with human thyroid microsomes coated onto microtitre plates. Similarly, a competitive radioassay was used to measure thyroid peroxidase (TPOAb), which involved the competitive inhibition of [^{125}I]-anti-TPO monoclonal antibody and sample autoantibody for the coated microsomes. The competitive radioimmunoassays demonstrated that TPOAb and MicAb were virtually identical, but they lacked the sensitivity and specificity of the corresponding immunoradiometric assay (IRMA) methods also investigated (Mariotti *et al.*, 1987).

Methods using monoclonal-assisted RIA, based on the competitive inhibition of the binding of [^{125}I]-labelled TPO (by sample autoantibody) to the solid-phase monoclonal TPOAb coated onto plastic tubes or micro-titre plates have also been described (Ruf *et al.*, 1988; Mariotti *et al.*, 1990). These methods were claimed to overcome the limitations of the previously described competitive radioimmunoassay, being sensitive, specific and easy to perform (Ruf *et al.*, 1988; Mariotti *et al.*, 1990). The measurement of MicAb and TPOAb using the competitive inhibition of TPO antigenic activity, provided assays of increased sensitivity as compared with measurement by passive haemagglutination (Ruf *et al.*, 1988; Mariotti *et al.*, 1990; Roti *et al.*,

1992). The method also obviated the need for large quantities of purified microsomal TPO, as required for an existing IRMA (Mariotti *et al.*, 1987; Ruf *et al.*, 1988).

However, a prerequisite for using a monoclonal antibody preparation is that it can compete effectively for the TPO binding, with virtually all TPOAb present in patients' sera [i.e. encompassing all the autoantibodies produced against each of the epitopes, as described in 1.4.1]. Massart *et al.* (1991) compared a competitive inhibition assay which was developed by Ruf *et al.* (1988) for measuring TPOAb, with a commercial IRMA MicAb kit (which used tubes coated with microsomal antigens and a preparation of Protein A labelled with [^{125}I] as antibody reagent) and showed that the assays were comparable in analytical reliability.

1.8.6 Immunoradiometric Assay (IRMA)

The conventional immunoradiometric (IRMA) (for antigen measurement) consisted of a two-step, non-competitive binding system in which the antibody was radioactively labelled instead of the antigen, and provided results directly proportional to the 'bound' radioactivity (i.e. complexed radiolabelled antibody) (Edwards, 1985). The measurement of autoantibody (i.e. MicAb/TPOAb) via IRMA would require labelled TPO antigen and an 'excess' amount of antibody reagent. The IRMA assay represented the use of another concept, i.e. immobilisation (covalent bonding) of antibody to a solid phase (Edwards, 1985).

There are a number of general ways in which IRMAs may be performed (i.e. the basic immunometric reaction, the 'two-site' method, and the indirect labelling [i.e the labelling of a second antibody] approach (Edwards, 1985). The IRMA used in this project, based on a method described by Beever *et al.* (1989) [see 2.4] for measuring TPOAb, was via the 'two-site' immunometric method, whereby the complex of radiolabelled TPO antigen and TPOAb was precipitated from the reaction mixture by the addition of a solid-phase antibody (magnetic particles coupled to Protein A), directed towards a

second antigenic binding site on the TPOAb. Protein A (SpA) is a 42 kDa cell wall constituent of *Staphylococcus aureus* (Potter *et al.*, 1996), and has a high affinity and high specificity for IgG (Bottomley *et al.*, 1995).

Although, reagent preparation is technically more demanding for the IRMA, it is often easier to perform (Edwards, 1985), and is frequently more precise with greater sensitivity than the competitive RIA and may be accompanied by enhanced specificity, but earlier methods required large amounts of the TPO antigen (Mariotti *et al.*, 1987). For optimal use in IRMAs, different reagents are often added sequentially, allowing time for each reagent to react and the excess removed before the addition of the next reagent. IRMAs almost invariably use solid-phase separation techniques to distinguish between the complexed or bound antibody and the unreacted or free antibody (Edwards, 1985). This makes the sequential additions technically very simple with the reduction of side-chain reactions, which could compromise the potential sensitivity and specificity of the assay system (Edwards, 1985). The IRMA can however, demonstrate the effects of 'non-specific' binding of the radiolabelled species, which can reduce the sensitivity of the assay. The washing of the solid-phase, radiolabelled TPO complex to remove any 'non-specific' interference can serve to enhance sensitivity.

The IRMA has a higher detection limit at high concentrations, giving overall a much wider working range, obviating the need for repeat analysis with dilution. The general, biphasic response (analagous to the 'prozone' effect in the precipitin test) is exemplified by the 'hook' effect in the immunoradiometric assay. The 'hook' effect takes place when the species being measured (i.e. TPOAb), is at a high concentration in reaction terms, and erroneously low results can be produced (Edwards, 1985).

Radioactive labelling provided assays with a very high level of sensitivity and reproducibility, which were amenable to automation for large scale processing (with the introduction of automated multi-well gamma counters for the detection of the low energy gamma-rays). However, the use of isotopes posed problems, including costly reagents with short shelf-lives, complex equipment for processing the results, and special safety measures to be

observed in the handling, storage and disposal of the reagents. These factors prompted the search for alternative, non-radioactive methodologies equivalent to their radioactive counterparts.

1.8.7 The Enzyme-labelled Assay

Enzyme-linked Immunosorbent Assay (ELISA)

The enzyme-labelled assay may be categorised into either immunoassay (limited reagent) or immunometric (excess reagent) techniques. Enzyme-immunoassays may be carried out without the use of separation techniques (i.e. homogeneous) (which is not applicable to radioactive labels) or with heterogeneous techniques, which utilise separation techniques (Edwards, 1985). Heterogeneous enzyme-immunoassays depend on the assumption that either an antigen [see 2.5] or antibody can be linked to an enzyme whilst retaining both immunological and enzymic activity in the resultant conjugate. As with radioimmunoassays, labelling the antigen with an enzyme, usually requires the availability of highly purified antigen (Edwards, 1985).

Enzyme-labelled immunometric assays using enzyme-labelled antibodies are invariably heterogeneous, requiring a separation stage, where the reacted enzyme-labelled component is separated by washing from the unreacted enzyme-labelled material, conventionally carried out with a solid-phase component. This basic immunometric assay, pioneered by Engvall *et al.* (1972) and Van Weemen & Schuurs, (1971) is usually referred to as Enzyme-linked immunosorbent assay (ELISA) and has been successfully applied to measuring large molecular weight substances (MWt over 10,000) such as antibodies in biological fluids, especially in blood. It was based on the principle that soluble antigens or antibody can be linked to an insoluble solid phase with the retention of reactivity of the immunological component (Voller *et al.*, 1979).

In the variant of ELISA used for measuring MicAb, the immunosorbent i.e. the microsomal antigen is attached to the solid phase. After reacting the immobilised antigen with the varying concentrations of antibodies present in

samples, and removal of unbound antibodies by washing, the bound antibodies are detected following a subsequent reaction with an enzyme-labelled anti-immunoglobulin antibody [see Figure 8 for a diagrammatic representation of the ELISA].

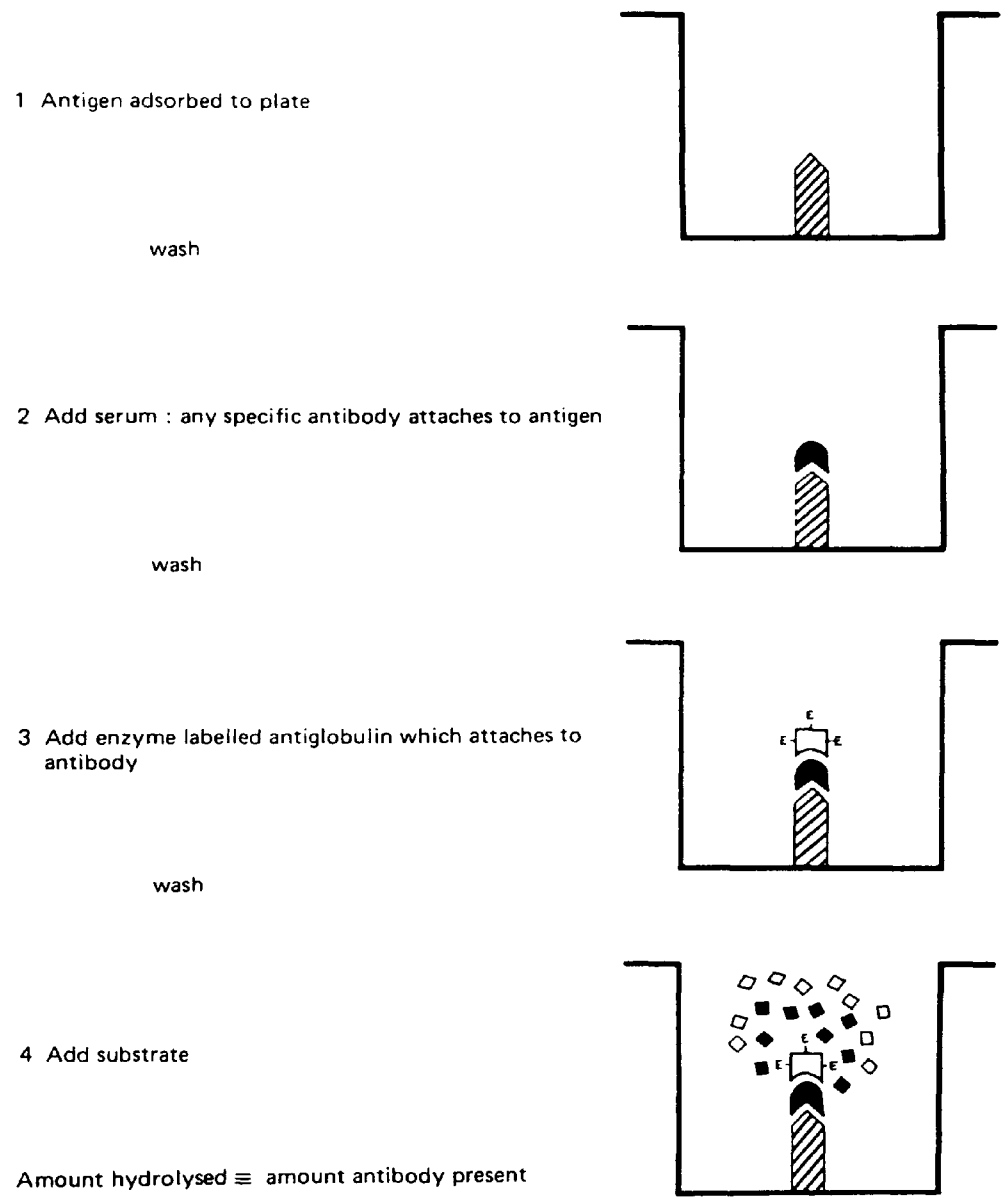


Figure 8 **The Indirect Method for Assay of Antibody** (Voller *et al.*, 1976: Bull. Wld. Hlth.Org.) Taken from 'The Enzyme-linked immunosorbent assay (ELISA): A guide with abstracts of microplate application' by Voller, Bidwell, & Bartlett, 1979, Nuffield Laboratories of Comparative Medicine, The Zoological Society of London, London.

Unlike in immunoradiometric assays, semi-purified antibodies, or even unpurified antibodies, have been used successfully in applications of the ELISA technique. However, this is probably a reflection of the fact that enzyme-labelled assays are generally not as sensitive as their radioisotope-labelled equivalents (Edwards, 1985).

ELISA consists of a series of incubations of different reagents separated by washing steps, which must be carried out in such a manner that there is no carry-over from one step to the next. The solid phase permits the separation of immunologically reacted from unreacted material during the test, and it is imperative that the solid phase should take up an adequate amount of the reactant in a reproducible manner. The variability at this stage is a major factor in determining the precision of all solid phase immunoassays (Voller *et al.*, 1979). Microplates made of polystyrene are easy to use and give adequate, reproducible uptake of most antigens merely by passive adsorption in alkaline solution (Miles *et al.*, 1998). The microplate format used in the ELISA is very convenient allowing the simultaneous washing of 96 wells, whereas the sequential washing of individual tubes, beads or discs can be very cumbersome by comparison. Fully automated ELISA systems, including washing arrangements are available, with the results being read photometrically, with rapid microplate readers capable of reading a whole ELISA plate in seconds (Voller *et al.*, 1979).

It is essential to include a reference positive and negative sample on each plate. The substrate reaction is stopped when the reference positive absorbance reaches a predetermined value. This enables valid comparisons to be made between tests on different occasions and in different laboratories.

The enzyme should have a high turnover rate of substrate product in order to provide a good final signal, and be minimally susceptible to interference from factors likely to be present in the sample e.g. enzyme cofactors (Edwards, 1985). The enzymes most favoured in the ELISA method have been alkaline phosphatase and in particular horseradish peroxidase, because of its low cost, easy conjugation and wide variety of substrates (Voller *et al.*, 1979).

In addition, a convenient substrate detector system must be available, which should be cheap, safe and easy to use. The chief requirement of a substrate is to provide a sensitive detection method for the enzyme in the conjugate. Ideally the substrate should initially be colourless which upon degradation gives a strong colour, and also produce completely soluble products with a high absorbance (Voller *et al.*, 1979). A wide variety of peroxidase substrates oxidised by H_2O_2 are available, but care must be taken to choose one with adequate solubility. ABTS (2-2'-azino-di-(3-ethyl benzothiazolin sulphone-6) (diammonium salt) has been used in ELISA (Groves *et al.*, 1990), and yields a final green colour with an A_{max} at 410 nm, but gives a poor dose response curve. Ortho-phenylenediamine dihydrochloride (OPD) is considered to be by far the best peroxidase substrate. The product is completely soluble and has a high absorbance at 492 nm, yielding an orange colour after stopping with H_2SO_4 [as used in the enzyme-immunometric assay described in 2.5]. OPD, however, is photosensitive so that reasonable care is required when it is used, and OPD along with some other peroxidase substrates has been reported as being mutagenic (Voller *et al.*, 1979).

ELISA can exhibit the 'high dose hook' effect as exemplified in the 'IRMA', i.e. when antigen levels are extremely high and give falsely low absorbance values. ELISA can also be prone to interference by other serum components (e.g. specific for IgG antibody), which become fixed to the sensitised solid phase giving false positive results (as evident in the agglutination assay) (Voller *et al.*, 1979). Thyroglobulin contamination of microsome preparations (still present after filtration) has also caused problems in designing an antimicrosomal antibody ELISA (Weetman *et al.*, 1983).

Many ELISA methods have been developed for measuring thyroid autoantibodies (Schardt *et al.*, 1982; Weetman *et al.*, 1983; Roman *et al.*, 1984; Groves *et al.*, 1990). A micro-ELISA method which measured MicAb and TgAb simultaneously was also described by Ng *et al.* (1987). Groves *et al.* (1990) demonstrated that the determination of TgAb and MicAb (TPOAb) by ELISA showed superiority in both speed and sensitivity over the traditional passive haemagglutination, but the introduction of ELISA into routine use

was initially hampered by the lack of primary standardisation, and different means of the quantitation and reporting of results (Groves *et al.*, 1990; Feldt-Rasmussen, 1996; Miles *et al*, 1998).

Autozyme™ TAB describes a commercial enzyme immunoassay (ELISA) for the screening and detection of autoantibodies against human thyroid peroxidase (TPO) in human serum. It employs horseradish peroxidase-conjugated anti-human antibodies and ABTS as its conjugate-substrate (A_{max} at 405nm), and is similar to the ELISA described by Groves *et al.* (1990). The Autozyme™ TAB Anti-TPO assay however, uses recombinant human thyroid peroxidase which does not contain contaminating thyroglobulin and/or mitochondria found in other microsomal antigen preparations.

Ng *et al.* (1987) described a combined enzyme immunoassay (micro-ELISA) technique for the simultaneous measurement of autoantibodies against thyroglobulin and thyroid microsome. It involved the use of an immuno-dot blot technique which was developed by Hawkes *et al.* (1982), and used nitrocelluose membrane filter discs and a conjugate consisting of anti-human immunoglobulin labelled with horseradish peroxidase, and diaminobenzidine as chromogenic substrate. The results correlated well with immunofluorescence and as is true of other conventional ELISA techniques, was found to be superior (i.e. more specific and sensitive) to haemagglutination (Ng *et al.*, 1987).

Heterogeneous enzyme-immunoassays combined the advantages of immunofluorescence and radioimmunoassay and overcame many of their disadvantages. Enzyme-labelled reagents are cheap to prepare, and are highly stable with long shelf lives. They also yield assays which approach the sensitivity of radioimmunoassay, and give objective results that can be determined, either visually, or with simple equipment (Voller *et al.*, 1979).

1.9 Chemiluminescent Immunoassay (CIA/ILMA)

On completion of this project, no references were cited in the world literature for the measurement of TPOAb/MicAb utilising a chemiluminescent label. Luminescence is defined as the emission of light resulting from the dissipation of energy from a substance in an excited state. Chemiluminescence is defined as 'a type of luminescence in which the light emission is caused by the products of a specific chemical reaction'. In response to the need for non-isotopic labels, interest was renewed in chemiluminescent molecules as an indicator of the antigen-antibody reaction. The phenomenon of light-emitting chemical reactions (chemiluminescence, CL) and (bioluminescence, BL) has been recognised for over 100 years. Although they have a diverse range of analytical applications, it is only in the last 10 years that they have made the transition from laboratory curiosities to core technologies and used in routine clinical laboratory investigations (Campbell, 1988).

Chemiluminescent molecules exploited as labels include luminol, isoluminol, acridinium esters, thioesters and sulphonamides, and phenanthridinium esters (Kricka, 1991). Various derivatives of luminol and isoluminol have been used for the chemiluminescent labelling of proteins. However, the quantum yield of emission of these compounds decreased after coupling, to produce labelled proteins of relatively low specific activity and sensitivity. The requirement of a catalyst in the reaction also led to a high chemiluminescent background and potential interference (Weeks *et al.*, 1983; Nelson & Kacian, 1990).

The basic acridine molecule was discovered as early as 1870 (Nelson & Kacian, 1990). Derivatisation of acridine at the 9 position (which possessed a quarternary nitrogen centre at the 10 position) yielded labile phenyl ester moities such as *N*-methyl acridinium esters (Weeks *et al.*, 1983). The *N*-methyl acridinium ester reacted with dilute alkaline hydrogen peroxide to yield *N*-methyl acridone in the excited state which then emitted light at 430 nm, in the form of a rapid flash [see Figure 9] (Weeks *et al.*, 1983). This chemiluminescence was elicited simply without the need for a catalyst for the

oxidation reaction to occur, thus alleviating problems encountered due to background and interference (Weeks *et al.*, 1983).

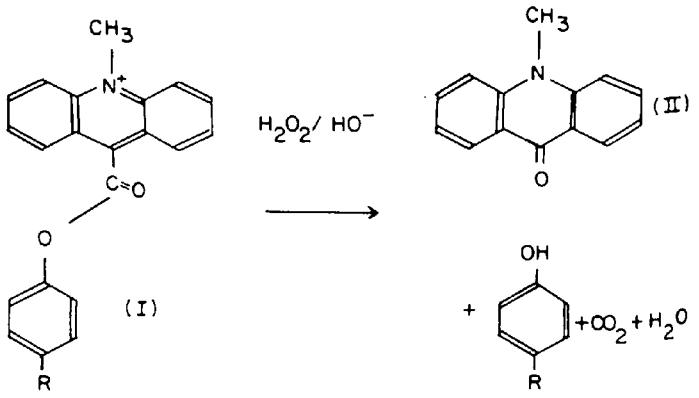


Figure 9 **Chemiluminescent reaction of acridinium esters**
I = Acridinium salt
II = N-methylacridone (Weeks *et al.*, 1983).

Weeks *et al.* (1983) reported the synthesis of a stable chemiluminescent acridinium ester derivative that was capable of spontaneous, covalent association, under mild, aqueous conditions with antibodies to yield stable, immunoreactive derivatives of high specific, chemiluminescent activity. The acridinium phenyl ester contains an *N*-hydroxysuccinimide (NHS) group which reacts specifically and covalently with primary amines, providing an easy way to directly label amine-containing compounds (Nelson & Kacian, 1990). This provided an easy and effective way for the direct and reproducible labelling of proteins (primary and secondary aliphatic amines) under aqueous conditions to predetermined specific activities which could be detected at lower concentrations than that of the corresponding [^{125}I] derivatives (Weeks *et al.*, 1983). The detection limit is approximately 5×10^{-19} mol (measured in a standard, commercially available luminometer), thus providing superior sensitivity to radiolabels, with detection linear to a concentration over more than 4 orders of magnitude (Weeks *et al.*, 1983; Nelson & Kacian, 1990).

The acridinium ester (AE) labels can be used in either a competitive, chemiluminescent immunoassay (CIA) or in the immunochemiluminometric (ILMA) ('sandwich') assay (Edwards, 1985). The ILMA can exhibit the 'high-dose hook' effect as is evident in its radioactive counterpart (IRMA).

The dissociative nature of the chemiluminescent reaction of this particular acridinium ester renders the emission characteristics of the molecule insensitive to microenvironmental changes (Weeks *et al.*, 1983). Assays are not prone to interference from many components of complex biological samples which can effect enzyme reactions, however, pH and temperature can influence luminescent reactions (Edwards, 1985). In the equilibrium reaction involving AE, the electronically excited *N*-methylacridone is formed in favour of pseudo-bases when at a low pH (approximately pH 5.0) (Weeks *et al.*, 1983).

The turbidity of the reaction is not usually a problem with a high tolerance of haemolysed, lipaemic and icteric samples evident, but interference from heterophilic antibodies and other autoantibodies (e.g. rheumatoid factor) still can present problems. More general interference can result from the effect of fluorescent lights in cuvettes or plastic tubes, and from minute amounts of impurities in the water, thus requiring maximally purified water supplies (Edwards, 1985).

The chemiluminescent AE can be detected rapidly and with high sensitivity (Edwards, 1985) and as the label reacts under mild conditions it has a universal application to protein immunoassay and represents a practical alternative to [^{125}I] (Weeks *et al.*, 1983). In chemiluminescent reactions, chemical energy generated as a result of the decomposition of a molecule produces excited-state intermediates that decay to a ground state with the emission of photons of light. The overall quantum yield of a CL reaction (the number of photons emitted/number of molecules reacting) is generally in the range 1-10% (Kricka, 1991). The photomultiplier tube (PMT), a photodetector, detects the photons of light emitted and converts them into electrical impulses (Parlett, 1978). The rapid reaction kinetics of acridinium esters result in improved sensitivity, since the short read time limits the

contribution of background noise and allows the reading of a large number of samples within a short time. A wide range of luminometers are now available for use for chemiluminescent reactions, including manual single-tube instruments and automated luminometers based on tubes, microtitre strips or plates as the reaction vessels (Kricka, 1991).

The AE labels avoid the hazards, restrictions and inconvenience of dealing with radioisotopes while affording equal or greater sensitivity, with subpicomolar detection limits. Reagents have good stability with shelf-lives up to six months stored at 4°C (Weeks *et al.*, 1983), and procedures are quick and simple and are amenable to automation (Kricka, 1991). With its Magic® Lite assays, [Bayer/Chiron Diagnostics (formerly Ciba Corning Diagnostics)] was the first to offer immunoassays utilising this chemiluminescent technology (Boland *et al.*, 1990) and now provides a vast array of clinical analyses via sophisticated immunoassay systems, with a method for the measurement of TPOAb, using paramagnetic particles as the solid phase and AE as the chemiluminescent label currently in its developmental stages.

1.10 The Aim of the Project

The aim of the study was to develop a non-radioactive immunoassay, which demonstrated comparable assay characteristics to its radioactive counterpart, and which could eventually be adapted to a more sophisticated, automated system, amenable to the screening for thyroid peroxidase autoantibodies (TPOAb) in human serum, to identify individuals with subclinical autoimmune thyroid disease (AITD) in 'high-risk' groups.

An attempt was made to develop an enzyme-immunometric assay and subsequently, three other immunoassays which utilised acridinium ester (AE), a highly sensitive and versatile chemiluminescent label. The thyroid peroxidase (TPO) used in these assays was derived from the microsomal fraction of the preparation described by Groves *et al.* (1990).

- **Method 1:** The immunoradiometric assay (IRMA) was based on a method described by Beever *et al.* (1989), and used a commercial preparation of TPO labelled with [^{125}I]. Magnetic particles coupled to Protein A were used to effect the separation of the final antibody-antigen radioactively labelled complex, which provided a direct measurement of the sample TPOAb.

- **Method 2:** The enzyme-immunometric assay involved the use of a preparation of microsomal TPO labelled with horse-radish peroxidase (HRP), and the magnetic Protein A suspension (which had been successfully applied as the separation phase in the IRMA). The final, spectrophotometic quantitation of TPOAb utilised the TPO-catalysed conversion of ortho-phenylenediamine (OPD), and provided a direct relationship with the sample TPOAb.

- **Method 3 (System 1):** This chemiluminometric assay used a microsomal preparation of TPO labelled with acridinium ester (AE). The separation medium consisted of magnetic particles coupled to anti-human IgG, with the final direct quantitation of TPOAb accomplished by the measurement of the AE [in relative light units (RLU)] attached to the magnetic particles.

- **Method 3 (System 2):** This competitive, assay system involved the use of a biotinylated preparation of microsomal TPO, and a purified preparation of human IgG (containing anti-TPO antibodies) labelled with AE. The separation of the acridinium-labelled complex was effected with streptavidin-labelled magnetic 'Dynabeads®', which exploited the biotin-streptavidin interaction, with the AE (measured in RLUs) being indirectly proportional to the sample TPOAb.

- **Method 3 (System 3):** This solid-phase system (which mimicked the ELISA), involved the immobilisation/coating of the microsomal TPO antigen onto plastic tubes. A commercial source of sheep anti-human IgG labelled with AE provided a direct quantitation of sample TPOAb via the me asurement of the RLUs attached to the tubes.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 Samples

Serum samples were obtained from patients, in which a normal (or borderline low) thyroxine (FT_4) result was accompanied by an unexplained elevated thyroid-stimulating hormone (TSH) level in routine thyroid function tests, which could indicate an underlying predisposition to developing hypothyroid autoimmune thyroiditis (HAT). Both FT_4 and TSH were measured via the Bayer/Chiron 'Chemiluminescent Immunoassay System' (ACS-180).

Samples were also obtained from patients with suspected autoimmune thyroid disease (AITD) upon clinical presentation and examination i.e. mainly Graves' disease (GD) or Hashimoto's thyroiditis (HT).

In addition, samples were obtained from patients visiting ante-natal clinics as part of the 'Wales Automated Follow-up Register' (WAFUR), in which female subjects were screened and monitored ante-natally and post-natally for post-partum thyroiditis (PPT).

A range of microsomal (TPO) autoantibody titres, including 20 'normal' titres [determined using an ELISA (Groves *et al.*, 1990) see Appendix 2:7] were studied. Samples (serum) were stored at -20°C prior to analysis (as recommended for long term storage) and to avoid microbial contamination (Miles *et al.*, 1998). Precautions were taken for the handling of human tissue samples in accordance with laboratory 'Health and Safety' guidelines.

2.2 Reference Material

2.2.1 Reference Material Used in the ImmunoradiometricAssay (IRMA) and the Enzyme-immunometric Assay

The 'Anti-thyroid Microsome Serum NIBSC Research Standard' (NIBS 66/387) was used (obtained from the 'National Institute for Biological Standards and Control', Hertfordshire, U.K.), which had an assigned nominal unitage of 1000 units per ampoule. The lyophilised contents were reconstituted in 1 ml of Tris HCl, pH 7.5 [see Appendix 1:1 for formulation],

and then 100 µl aliquots were 'snap-frozen' to -50°C in solid CO_2 and isopentane and stored at -20°C. The stock, standard aliquot was diluted 1 in 20, and further 1 in 10 dilutions were made to provide standard concentrations of 1000, 100, 10, and 1 U/L with the blank consisting of Tris HCl, pH 7.5.

2.2.2 Reference Material Used in the Chemiluminescent Immunoassays and the Enzyme-linked Immunosorbent Assay (ELISA)

An 'in-house' reference material was used which consisted of pooled serum samples of high microsomal thyroid peroxidase autoantibody (MicAb/TPOAb) activity, which had been calibrated against the 'Anti-thyroid Microsome Serum NIBSC Research Standard' (NIBS 66/387). The stock standard (20,000 kIU/L) [solubilised with 1% deoxycholic acid (DOC)] was diluted with phosphate-buffered saline (PBS), pH 7.5 [Appendix 1:5] to give concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 kIU/L (kIU/L ≅ U/L described previously).

2.3 Quality Control

The quality control used in the immunoradiometric assay (IRMA), (Method 1), consisted of serum from patients who had 'normal' and 'high' titres of microsomal autoantibody (MicAb) as determined by indirect agglutination using the Serodia® - AMC kit , Fujirebio INC. [Appendix 2:8].

Similarly, serum of known 'normal' and 'high' MicAb activities [assayed by the established ELISA methodology used by Groves *et al.* (1990)] were used in the chemiluminescent immunoassays (Method 3: Systems 1, 2 and 3) and the ELISA. These controls were stored in frozen aliquots prior to analysis and were diluted by a factor of 100 for analysis with PBS, pH 7.4. The high control 'L4' used (also derived from patient serum), was stored in aliquots (diluted 1:10 with PBS, pH 7.4), which required a further 1:100 dilution for use.

Unless stated otherwise, all chemicals were purchased from 'British Drug House Ltd., (BDH), Poole, Dorset., U.K. [see Appendix 4 for the list and addresses of suppliers], and were handled in accordance with the 'COSHH' (Control of Substances Hazardous to Health) regulations.

2.4 Method 1

Immunoradiometric Assay (IRMA) Using a Commercial Preparation of Thyroid Peroxidase (TPO) Labelled with [^{125}I] and Magnetic Particles Coupled to Protein A

2.4.1 Principle of Assay

The diluted serum sample containing the microsomal (TPO) autoantibody (MicAb/TPOAb) to be measured was incubated with the [^{125}I]-radioactively labelled TPO, resulting in the formation of antibody-antigen labelled complexes. The magnetic solid-phase consisted of magnetic particles coupled to Protein A (MPA) which effected the separation of the labelled complex and the immobilised radioactive counts were measured. The counts were directly proportional to the sample autoantibody concentration [see Fig 10].

2.4.2 Thyroid Peroxidase (hTPO)-[^{125}I] Label

A commercial preparation of human thyroid peroxidase (hTPO), radioactively labelled with [^{125}I] was used (obtained from RSR Ltd., Avenue Park, Pentwyn, Cardiff., U.K.). The lyophilised material required reconstitution with 5 ml of Tris HCl buffer, pH 7.5 (Beever *et al.*,1989) giving a specific activity of 8 Kbq/ml.

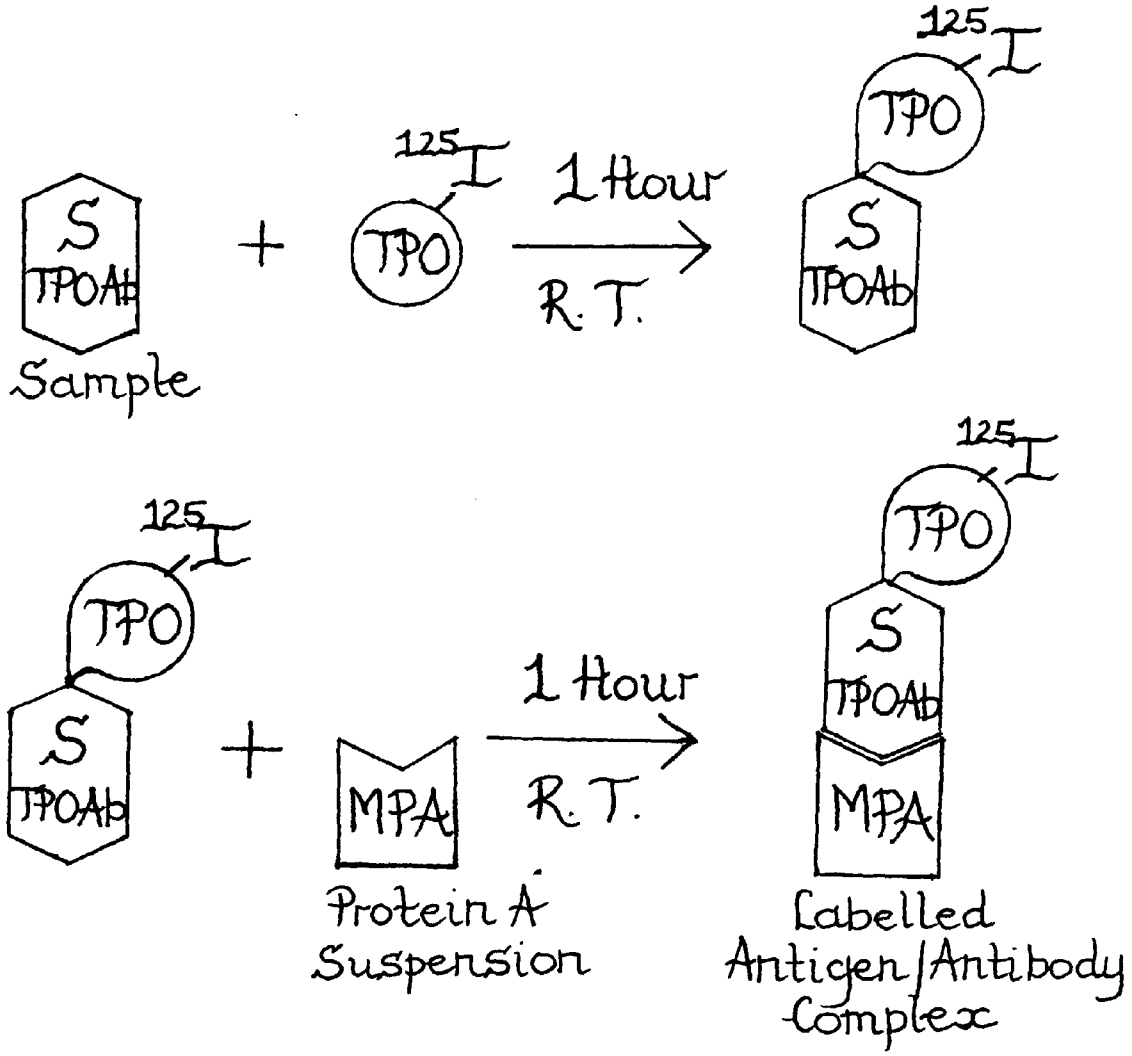


Figure 10 Diagrammatic Representation of the Immunoradiometric Assay (IRMA).

The radioactive counts attached to the final Antigen/Antibody/Protein A complex in this non-competitive immunoassay are directly proportional to the sample thyroid peroxidase autoantibody concentration (TPOAb).

2.4.3 Protein A Suspension

Procedure for Coupling Magnetic Particles to Protein A

The preparation of the solid-phase was based on that used in an 'in-house' ferritin immunoradiometric assay which involved the coupling of BioMag® 4100 magnetic particles to a mouse anti-serum (i.e. anti-ferritin antibody).

BioMag® 4100 magnetic particles (Advanced Magnetics Inc., Cambridge., U.K.) consisted of a suspension of magnetic iron oxide particles coated to provide primary amino groups for the covalent attachment of proteins.

Well-mixed BioMag® magnetic particles (6 ml), were washed twice, by adding 20 ml of coupling buffer (0.1M phosphate buffer, pH 7.4), [Appendix 1:1], and thoroughly mixed on a roller mixer for 30 minutes.

The preparation was centrifuged and the supernatant carefully removed and the pellet resuspended in 12 ml of 0.1M phosphate buffer, pH 7.4 and 12 ml of 5% glutaraldehyde (stock 25% glutaraldehyde reagent diluted with double-distilled water) and then mixed 'end-over-end' overnight at room temperature. (The 5% glutaraldehyde solution was added in order to activate the BioMag® magnetic particles for coupling to the Protein A). The mixture was centrifuged, the supernatant aspirated to remove any unreacted glutaraldehyde and the particles were resuspended in 20 ml of coupling buffer. The preparation was then mixed 'end-over-end' for 1 hour, centrifuged and the supernatant removed. This wash procedure was repeated five times.

To the pellet of 4 ml of washed magnetic particles, 2.5 ml of the Protein A solution (*Staphylococcus aureus* / Extracellular P.6031) (Sigma-Aldrich Co. Ltd., Dorset., U.K.) was added. This was prepared by dissolving 1 mg in 5 ml of 0.1M phosphate buffer, pH 7.4). The particles were gently resuspended, and the contents were mixed 'end-over-end', overnight at room temperature. (N.B. A 200 µl aliquot of the Protein A solution had been removed in order to estimate its protein content prior to coupling).

To protect reactive groups and remove uncoupled protein, the particles were washed in 0.1M phosphate buffer, pH 7.4, and then resuspended in 20 ml of

0.1M phosphate buffer, pH 7.4 containing 0.3 g (0.2M) glycine (quenching solution), and mixed 'end-over-end' for 2 hours at room temperature. The protein content of this supernatant was measured together with the aliquot of the pre-coupling supernatant in order to calculate the percentage uptake of Protein A by the magnetic particles and determine the coupling efficiency. (A micro-method for the determination of protein in cerebrospinal fluid and urine, using Ponceau S as the stain was used (Pesce *et al.*, 1973), [see Appendix 2:2 (b) for details on procedure].

The particles coupled to Protein A were washed 3 times each with 20 ml of 0.1M phosphate buffer, pH 7.4 containing 1M and then 0.15M sodium chloride (NaCl) respectively, in order to remove or reduce non-covalently adsorbed protein on the magnetic particles.

The particles were resuspended in 5 ml of 0.1M phosphate buffer, pH 7.4 and then appropriately diluted in the same buffer for use. The particles were stored at 2-6°C [see Appendix 2:1 for further details on the BioMag® 4100 magnetic particles].

2.4.4 Immunoradiometric Assay Procedure

Aliquots (50 µl), of the TPO autoantibody standards (i.e. 0, 1, 10, 100 and 1000 U/L) [see 2.2.1], or quality control diluted 1:100, [see 2.3] were incubated in plastic 5 ml Sarstedt tubes for 1 hour at room temperature, with 50 µl of the reconstituted [^{125}I]-labelled TPO [see 2.4.2]. The Protein A Suspension (100 µl) (diluted to an optimal concentration ratio of 1:5, with 0.1M phosphate buffer, pH 7.4) [see 2.4.3] was added to each tube and then incubated for a further 1 hour at room temperature. The final magnetic, radioactively-labelled antibody complex was precipitated out in the pellet and separated using a magnetic rack. The pellet was then washed with the addition of 1 ml of the Tris HCl buffer, pH 7.5, magnetised down and the final supernatant decanted. The radioactivity in the final pellet of magnetic particles was counted for 2 minutes using the LKB: Wallac 1260 Multigamma Counter System and the RIACALC software package.

Various experiments were carried out to determine optimum assay conditions as described in 3.1.

2.4.5 Determination of the Assay Sample Recovery

The recovery of the assay was determined by diluting a serum sample (which contained a high titre of microsomal autoantibody) 20-fold to 20,000-fold with Tris HCl, pH 7.5 and assayed (as described in 2.4.4), with the Protein A suspension diluted in the ratios of 1:5 and 1:2.5. The results of the sample dilutions were then interpolated from the sigmoidal standard curve and the logarithmically converted data plotted against the sample dilution ratio.

2.4.6 Comparison of IRMA with Indirect Agglutination

Ten samples (diluted 1:20 and 1:50, using the Tris HCl diluent, pH 7.5) were assayed with the magnetic particles diluted in a ratio of 1:5 (using the assay procedure described in 2.4.4). The results were compared with those obtained via indirect agglutination (Serodia®-AMC).

2.5 Method 2

Enzyme-immunometric Assay Using Microsomal TPO Labelled with Horseradish Peroxidase (HRP) and Magnetic Particles Coupled to Protein A

2.5.1 Principle of Assay

The TPO labelled with horseradish peroxidase (HRP-TPO) reacted with the TPOAb in the sample to form an enzyme-labelled-antigen-TPOAb complex. Magnetic particles coupled to Protein A (MPA) were used (as in the IRMA, Method 1) in order to effect the separation of this final complex. The final quantitation involved the reaction of HRP (labelled with TPO), with o-phenylenediamine dihydrochloride (OPD), which produced an orange colour and was measured colorimetrically at 492 nm, the final intensity of which being directly proportional to the sample (microsomal) thyroid peroxidase autoantibody (TPOAb) concentration [see Fig 11].

2.5.2 Horseradish Peroxidase / Thyroid Peroxidase Label (HRP-TPO)

Procedure for Labelling TPO with Horseradish Peroxidase (HRP)

Thyroid microsomes (solubilised with 1% deoxycholic acid) [Appendix 2:4] were coupled with HRP via the 'Periodate' method as described by Wilson & Nakane (1978), which was claimed to produce a higher yield of label as compared with coupling procedures employing glutaraldehyde (Voller *et al.*, 1979). Sodium periodate, a cross-linking agent, was employed to activate the peroxidase enzyme for labelling, to produce a HRP-TPO label which affords a maximum retention of reactivity of both the HRP and TPO. The aim was to couple 3 nmoles of HRP to 2 nmoles of TPO.

HRP (3.6 mg) (Sigma-Aldrich Co. Ltd., Dorset., U.K.), was dissolved in 0.6 ml of double-distilled water; (a 100 µl aliquot of this preparation was removed and frozen at -20°C, for future estimation of the pre-coupling activity of the HRP). A freshly prepared solution of 0.1M sodium periodate (100 µl) was

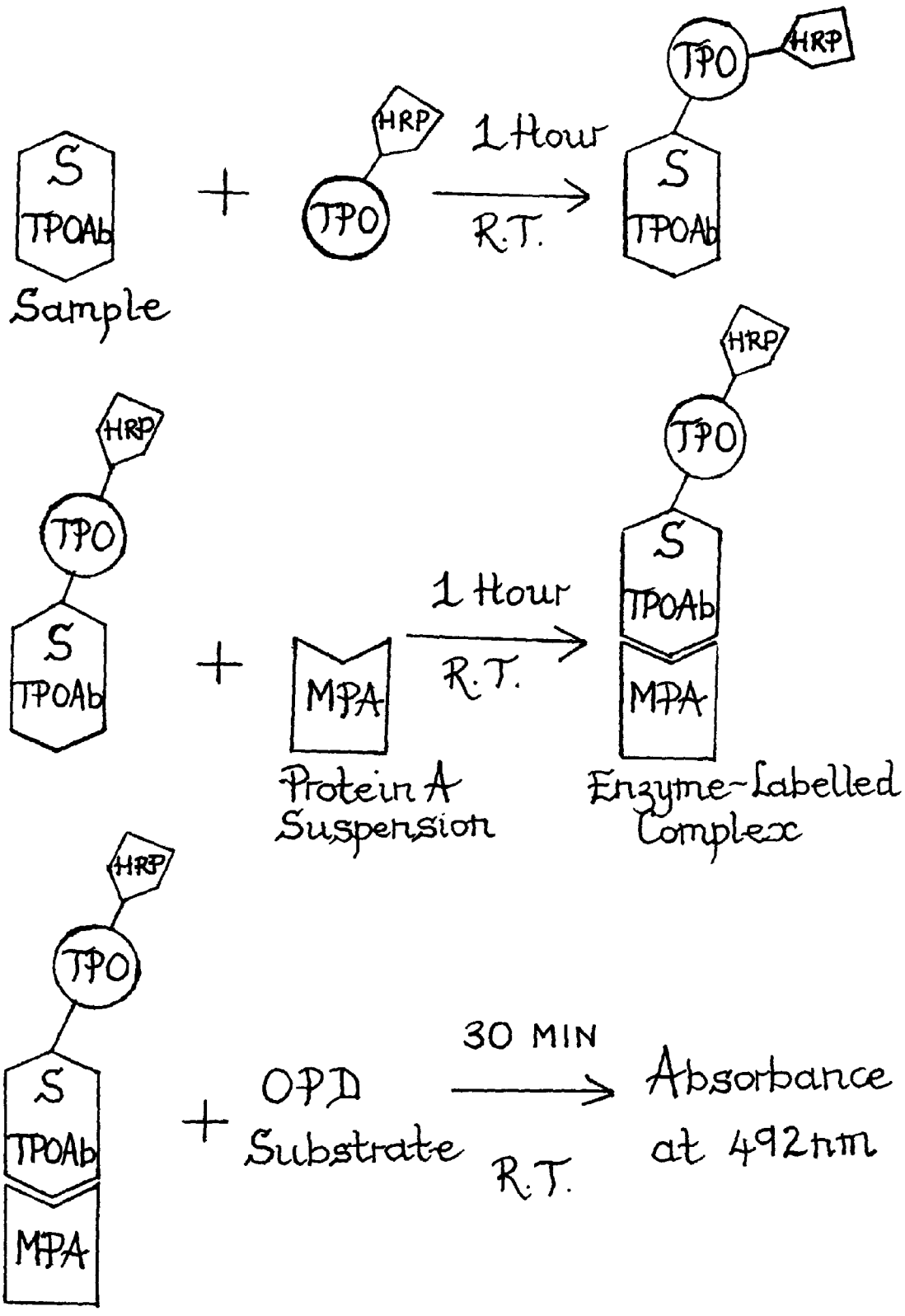


Figure 11 Diagrammatic Representation of the Enzyme-immunometric Assay. The final absorbance at 492 nm being directly proportional to TPOAb-S in this non-competitive immunoassay.

added to the remainder of the HRP dilution (giving a HRP concentration of 5 mg/ml), and was mixed for 30 minutes at room temperature.

The mixture was then dialysed overnight at 4°C (in visking, dialysis tubing; 8/32: Size: 1: Medicell International Ltd.,) against 1 litre of 1mM sodium acetate buffer, pH 4.4 [Appendix 1:2]. Then, 1.4 ml of 0.2M carbonate buffer, pH 9.5, containing 0.4% deoxycholate [Appendix 1:2], was added to the dialysate, and 100 µl of this was added to 200 µl of the solubilised TPO. The mixture was mixed 'end-over-end' at room temperature for 2 hours.

A fresh preparation of 0.1M sodium borohydride (10 µl), was then added, in order to inactivate any uncoupled peroxidase and this was left to stand at room temperature for 2 hours at 4°C. The mixture was then dialysed twice against 1 litre of 0.5M phosphate buffered saline (PBS), pH 7.4, containing NaCl (0.09%) and sodium deoxycholate (0.4%), [Appendix 1:2]. Finally, 40 µl of a 1% solution of human serum albumin (Sigma-Aldrich Co. Ltd., U.K.) (stock solution diluted in 0.5M PBS, pH 7.4), was added to the dialysate, to block any free binding sites and the label was stored at 4°C prior to use.

2.5.3 Protein A Suspension

The Protein A suspension (MPA) was as used in the immunoradiometric assay [see 2.4.3 for preparation details].

2.5.4 Enzyme-immunometric Assay Procedure

The NIBSC standardised TPO auto-antibody was diluted (as in the IRMA, Method 1, using 0.5M PBS, pH 7.4), to give the concentrations (0, 1, 10, 100, 1000 U/L). Each dilution (50 µl), was incubated for 2 hours at room temperature in the dark with 50 µl of the HRP-TPO label (diluted 1 in 200 with 0.5M PBS, pH 7.4).

Then, 100 µl of Protein A suspension (diluted in a ratio 1:2.5 with 0.1M phosphate buffer, pH 7.4) (as used in Method 1), was added and further incubated in the dark for 1 hour at room temperature. The magnetic particles

were separated and washed with 1 ml of 0.1M phosphate buffer, pH 7.4, and the supernatant removed. Then, 1 ml of freshly prepared OPD substrate reagent [o-phenylenediamine dihydrochloride and 10 µl of hydrogen peroxide in phosphate/citrate buffer, pH 5.0], was added to the particles, (substrate reagent as used by Voller *et al.*, 1979) [Appendix 1:2], and incubated at room temperature in the dark for 30 minutes. The reaction was stopped by the addition of 50 µl of 10M NaOH and the orange supernatant removed, the intensity of which was measured colorimetrically at 492 nm.

2.6 Method 3 (System 1)

Non-Competitive Chemiluminometric Assay Using TPO Labelled with Acridinium Ester (AE)

2.6.1 Principle of Assay

This assay was based on the same principle as Methods 1 and 2, but instead a chemiluminescent label was employed, i.e. acridinium ester [4-(2-succinimidyloxycarbonylethyl)phenyl-10-methylacridinium-9-carboxylate fluorosulphonate] to label the microsomal TPO. Magnetic particles coupled to anti-human IgG (MAb-Anti-IgG) were used to effect the separation of the antigen-antibody complex labelled with acridinium ester. The *N*-methyl acridinium esters react with hydrogen peroxide in base to yield *N*-methyl acridone in the excited state which then emits light at 430 nm, measured as Relative Light Units (RLU) [see Figs 9 & 12].

2.6.2 Thyroid Peroxidase / Acridinium Ester Label (Acrid-TPO)

Procedure for Labelling TPO with Acridinium Ester

The TPO protein [prepared as described by Groves *et al.* (1990)] [Appendix 2:4] was labelled using a kit from Molecular Light Technology Research Limited, Cardiff, U.K. Varying ratios of acridinium ester (AE) to TPO protein content were investigated in the labelling procedures, and a comparison between crude preparations and those solubilised with 1% deoxycholic acid was made.

The TPO preparation was diluted in labelling buffer [Appendix 1:3] to give a protein content of 150 µg/ml. The protein preparation (30 µg), was added to a vial which contained 5 µg of succinimidyl activated AE and the contents were mixed well and incubated for 15 minutes at room temperature in the dark (as the activated AE is photosensitive).

Then, 100 µl of quenching buffer (DL-lysine monohydrochloride, 10 mg/ml) [Appendix 1:3] was added to the vial and gently mixed and stored in the dark, at room temperature for 5 minutes in order to 'quench' unreacted label. The

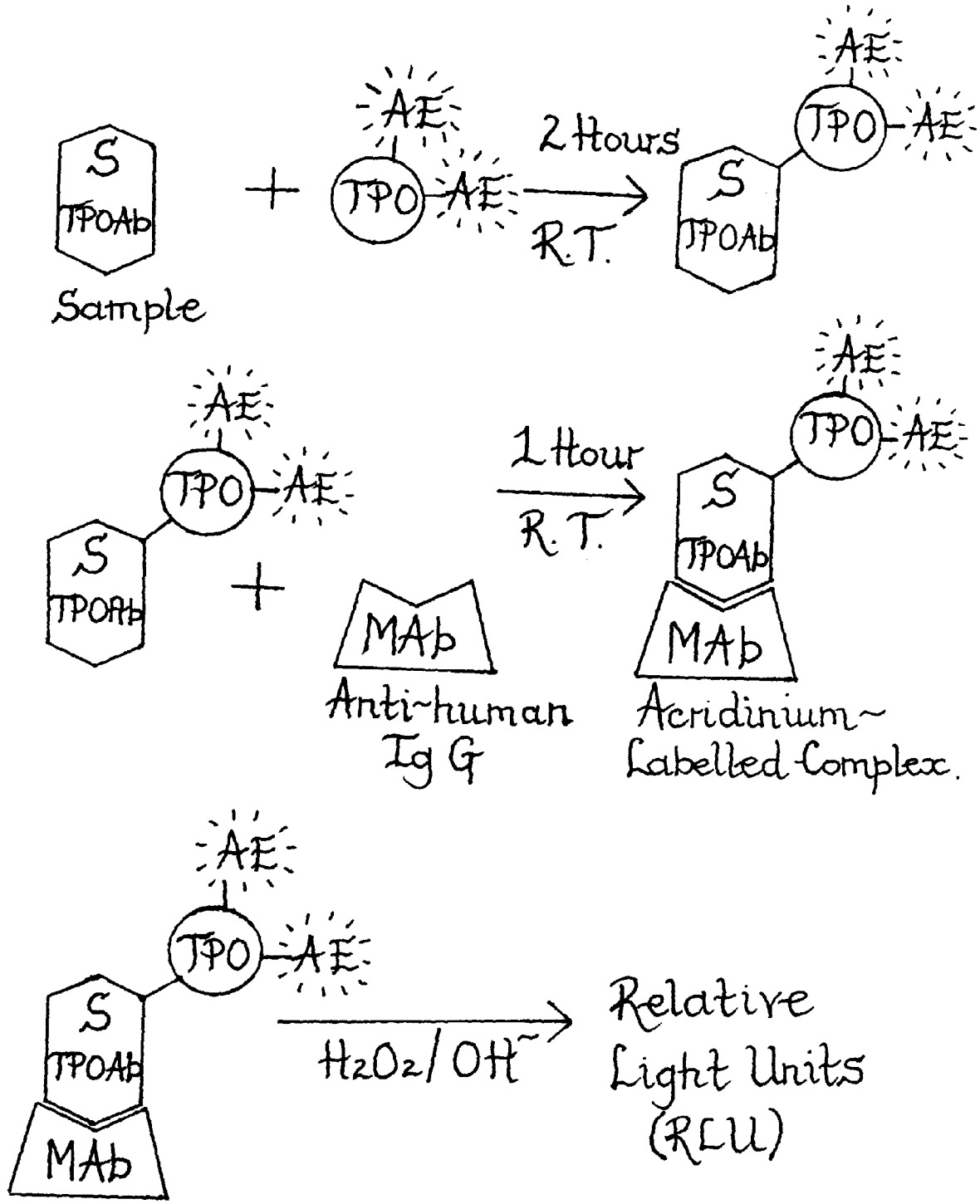


Figure 12 Diagrammatic Representation of the Chemiluminometric Assay (System 1)

The acridinium ester (AE) (in RLUs) attached to the final magnetic complex are directly proportional to sample thyroid peroxidase autoantibody (TPOAb-S) in this non-competitive immunoassay.

quenching buffer was subsequently removed during the gel filtration purification process [see 2.6.4].

Following this, 30 µl of a 10% solution BSA in 0.1M phosphate buffer, pH 8.0, was added to the vial in order to block any existing free sites and reduce non-specific binding of interfering proteins. The protein/label mix was stored at 4°C prior to loading onto the column.

2.6.3 Varying the Ratio of Acridinium Ester to TPO in the Labelling Procedure

Varying concentrations of AE to TPO protein content were obtained, by diluting the contents of one vial with 100 µl of acetonitrile (methyl cyanide). An appropriate aliquot was removed to give the required percentage/concentration [i.e. 10% (0.5 µg) and 50% (2.5 µg)] and transferred into a small glass vial and concentrated down. The diluted TPO protein was then added and the labelling procedure continued (as described in 3.6.2). For 1% of a vial, (i.e. 0.05 µg of AE), 10 µl of a vial (diluted with 100 µl of acetonitrile) was taken and further diluted with 90 µl of acetonitrile. A 10 µl aliquot was then removed and concentrated down and the diluted TPO protein was then added and the labelling procedure continued.

2.6.4 Purifying the Acridinium/TPO Label Using Gel Filtration

Sephadex™ G25 (Medium) (Pharmacia Fine Chemicals) was hydrated according to the manufacturer's instructions and a column of 10 ml volume prepared. The column was equilibrated with 30 ml of elution buffer [Appendix 1:3]. The AE-labelled TPO was applied to the top of the column and fifteen 1 ml aliquots of eluate were collected. Each fraction was mixed, and 10 µl of each was diluted with 1 ml of elution buffer. Then, 10 µl of this dilution was transferred to plastic tubes and the total Relative Light Units (RLUs) in each tube were measured using the Bayer/Chiron Magic® Lite Chemiluminescent Luminometer. This involved the addition of 300 µl of hydrogen peroxide solution which oxidises the ester, followed by the addition of 300 µl solution

of sodium hydroxide which was added to adjust to the necessary reaction pH, accelerating the oxidation process. The light energy emitted (measured in RLUs) at 430 nm was proportional to the acridinium content.

The fractions which contained the labelled TPO (i.e. with the highest RLUs signals) were retained for use. (The fractions collected were the ones on the ascending side of the elution peak, which would hopefully provide a label of a high purity and specificity). The fractions were then stored in the dark at -20°C.

2.6.5 Magnetic Separation Phase

The separation phase consisted of magnetic particles coupled to anti-human IgG (MAb-Anti-IgG) [see Appendix 2:5 for preparation].

2.6.6 Chemiluminometric Assay : System 1

The 'in-house' MicAb/TPOAb standard (calibrated against the NIBSC Standard) was diluted with assay diluent (PBS, pH 7.4), to give final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 kIU/L. The control sera and patient samples were diluted by 100 with assay diluent. Aliquots, each of 100 µl of the diluted standards, controls and patient sera were incubated in 5 ml Sarstedt plastic tubes, with 100 µl of TPO labelled with AE [diluted 1:2000 with label diluent (i.e. PBS, pH 7.4 containing 1% sheep serum)]. The sheep serum was added to reduce the effects of non-specific adsorption to the plastic tubes or other support in the assay system. The tubes were vortex mixed and then incubated for 2 hours at room temperature.

Then, 500 µl of the magnetic antibody suspension (MAb-Anti-IgG) (washed 4 times with assay diluent) [Appendix 2:5] was added to each tube. [The stock preparation of MAb-Anti-IgG was diluted by 20 with assay diluent (PBS, pH 7.4) for use]. The tubes were vortex mixed and further incubated for 1 hour at room temperature.

The magnetic particles were then separated, decanted and washed four times using the ELISA wash solution [Appendix 1:5], (using a Bayer/Chiron magnetic separator). The RLUs associated with each pellet was measured as described previously [see 2.6.4]. The logarithmically transformed data (log_{10} RLU versus log_{10} TPOAb standard concentration) was plotted using the Excel Version 5 Statistical Software Package.

2.7 Method 3 (System 2)

Competitive Immunoassay Using TPO Labelled with Biotin, Human IgG Labelled with Acridinium Ester and Magnetic Beads Coupled to Streptavidin

2.7.1 Principle of Assay

System 2 involved the labelling of an antibody to TPO from a patient sample with a high anti-TPO titre with acridinium ester. This was incorporated into an assay system using TPO labelled with biotin and a separation stage using magnetic beads coupled to streptavidin.

In this system there was competition between the thyroid peroxidase autoantibody (TPOAb) in the sample and the acridinium-labelled-IgG (Acrid-IgG), for the biotinylated TPO (Biotin-TPO). The higher the TPOAb in the sample, the less Acrid-IgG will be bound to the Biotin-TPO, and subsequently to the streptavidin beads. The separation of the Biotin-TPO-antibody-acridinium complexes was effected via the magnetic beads labelled with streptavidin, with the sample TPOAb concentration being inversely proportional to the final RLUs of the acridinium ester attached to the beads [see Fig 13].

2.7.2 (i) Biotinylation of Microsomal Thyroid Peroxidase (TPO) in Solution Using the ECL Protein Biotinylation Module

The ECL Protein Biotinylation Kit was supplied by Amersham Life Science and had the capability of labelling primary amino groups on proteins using an N-hydroxysuccinimide-biotin ester. The high affinity binding of the small water soluble vitamin biotin, to the bacterial streptavidin was then exploited to effect the separation of the biotin-labelled immune complex.

A 100 µl aliquot of crude microsomes (10mg/ml) was diluted with 900 µl of coupling buffer (800mM bicarbonate buffer, pH 8.6). Deoxycholic acid (DOC) (1%), was added to the crude TPO dilution in order to solubilise the cell membranes to avoid aggregation. The solubilisation of the crude TPO

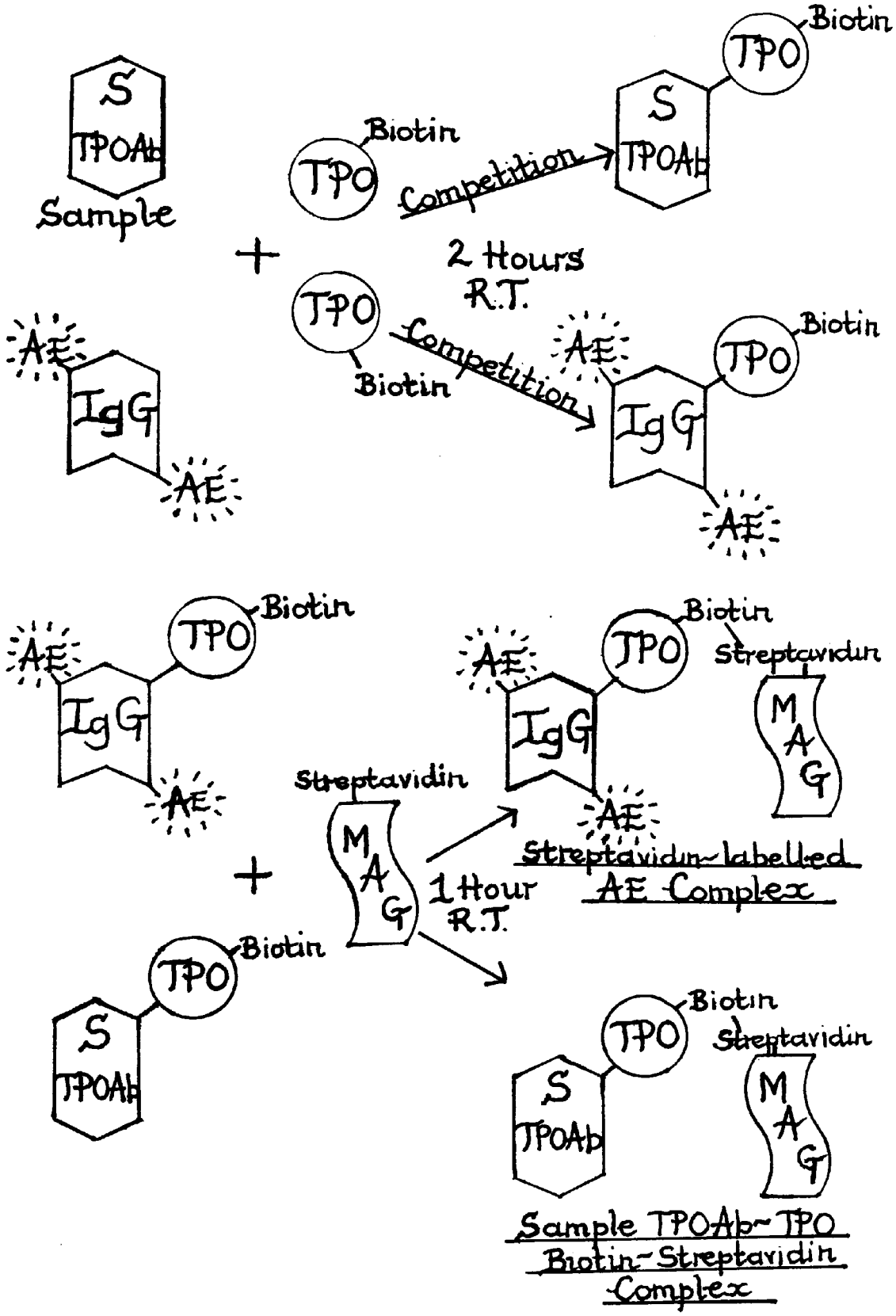


Figure 13 Diagrammatic Representation of the Chemiluminescent Immunoassay (System 2). The AE attached to the Biotin-TPO- Acrid-IgG-Streptavidin-labelled complex is inversely proportional to sample thyroid peroxidase autoantibody (TPOAb).

causes the formation of micelles which results in the charged groups of the proteins being relocated on the inner of the micelles, thus eliminating any aggregation. Biotinylation reagent (40 µl), (equilibrated to room temperature before use), was slowly added via a syringe to the 1 ml of diluted protein (40 µl of biotin reagent is required per 1 mg of protein). The biotin reagent (supplied ready for use in the kit) was added centrally to avoid contact wth the plastic tube.

The biotin/TPO solution was mixed for 2 hours at room temperature, and a further 1 ml of coating buffer was added, (reducing the TPO protein concentration to 0.5 mg/ml), and providing 2 ml of biotin/TPO protein solution, the minimum volume that could be used in this biotinylation procedure.

2.7.2 (ii) Purification of the Biotinylated TPO (Biotin-TPO) via Gel-Filtration Using Sephadex™ Gel 25

The 5 ml columns containing Sephadex™ Gel 25 were supplied ready for use with the ECL Biotinylation kit. The 2 ml of the biotin/TPO solution was carefully applied to the column which had been equilibrated with 5 ml of PBS / 1% BSA and then washed with 20 ml PBS, pH 7.5 [Appendix 1:4].

The Biotin-TPO label was eluted off the column, with 5 ml of PBS buffer, pH 7.5, and the eluate was collected in 1 ml fractions. UV absorbance at 280 nm verified the fraction number in which the eluted protein was present. The Biotin-TPO label was eluted in the first 3 ml which agreed with the manufacturers findings. The unbound biotin was also removed by gel filtration as the presence of 'free' biotin will interfere in the interaction of the streptavidin beads with the Biotin-TPO label. The two fractions containing the Biotin-TPO label were then stored at 4°C, with the addition of sodium azide (0.02%).

2.7.3 Labelling of Purified IgG Sample (Containing A High Level of Microsomal Autoantibody) with Acridinium Ester (AE)

A sample containing high microsomal antibody activity (determined by ELISA), was purified via the salting out procedure using ammonium sulphate [Appendix 2:6]. The purified IgG sample (2 mg/ml), was appropriately diluted to give a final concentration of 50 µg of immunoglobulin G (IgG) in a volume of 200 µl, (the concentration recommended by 'Molecular Light Technology Research Limited' for the labelling procedure). The 200 µl aliquot of sample was then added to a vial containing the acridinium ester (AE) (5 µg), and the same labelling and purification procedure was carried out as for the preparation of the Acrid-TPO label (described in 2.6.2 & 2.6.4). The 1 ml aliquots containing the label were then stored frozen at -20°C prior to analysis.

2.7.4 Preparation of the Dynal 'Dynabeads®'

Dynabeads® M-280 Streptavidin (obtained from 'Dynal' U.K. Ltd.,) consisted of beads covalently labelled with streptavidin, a protein of molecular weight (MWt) 66,000 consisting of 4 identical subunits which have a high affinity for biotin (Kd = 10^{-15}M). The superparamagnetic, polystyrene beads were supplied as a suspension containing a $6.7x10^{8}$ concentration of Dynabeads® / ml (10 mg/ml), dissolved in PBS, pH 7.4, containing 0.1% BSA and 0.02% sodium azide.

The Dynabeads® were gently resuspended to obtain a homogeneous suspension and were washed before use to remove the sodium azide preservative. The appropriate volume required for the assay was removed and transferred to a plastic tube and placed on the Dynal MPC (magnetic separator). The supernatant was removed and the beads resuspended in 2 ml of PBS, pH 7.5, and washed on the roller-mixer for 15-30 minutes. The particles were magnetised down and the supernatant removed and the wash procedure repeated. The particles were separated and resuspended in PBS, pH 7.5 diluent to the original volume and were ready for use.

An equivalent of approximately <1 µl of biotin solution (assuming 100% recovery of biotin in the 2 fractions after gel filtration) would be in a 100 µl aliquot of the Biotin-TPO label (diluted 1:100). Thus, using this rough estimation, 10 µl of beads per assay tube should be sufficient and in excess. ['Dynal' quote an uptake ratio of 1 (mg/ml) of streptavidin beads to 4 (mg/ml) of biotin].

2.7.5 Assay System Using Biotin-TPO and Human Anti-TPO Labelled with Acridinium Ester (AE)

Standards and quality control (100 µl aliquots), [diluted with PBS as in Method 3 (System 1)], were pipetted into plastic Sarstedt tubes. To each tube 100 µl of Biotin-TPO label [diluted accordingly with (TBS)/1% sheep serum], plus 100 µl of IgG labelled with AE (Acrid-IgG) (diluted appropriately with PBS/1% sheep serum, as described later) was added, the contents vortex mixed and incubated at room temperature for 2 hours.

The washed Dynabeads® (M-280 Streptavidin) (10 µl), were added to each tube, vortex mixed and further incubated for 1 hour at room temperature. The beads were then separated from the supernatant using a Bayer/Chiron Magnetic Rack and the supernatant decanted. The beads were washed and decanted four times using ELISA wash solution [Appendix 1:5] and the RLUs of the acridinium ester attached to the beads were measured via the luminometer as before.

Standard curves were set up using the Biotin-TPO label diluted 1:1000 and 1:2000, with the Acrid-IgG label initially diluted 1:2000. A series of dilutions of the high 'L4' control were also assayed using the Biotin-TPO diluted 1:10, 1:100 and 1:1000 and the Acrid-IgG label diluted both 1:1000 and 1:2000.

Results indicated a problem with the Acrid-IgG label, therefore it was decided to further purify the preparation using affinity chromatography, as described below.

2.7.6 Affinity Chromatography Using Cyanogen Bromide (CNBr)-Activated Sepharose™ 4B Gel

2.7.6 (i) Preparation of CNBr-Activated Sepharose™ 4B Gel Column

CNBr-activated Sepharose™ 4B provides the most convenient way to immobilise ligands containing primary amino groups, by the cyanogen bromide method. The coupling reaction is spontaneous, rapid, safe and easy to carry out and the amount of coupled ligand can be reproducibly controlled (Pharmacia Fine Chemicals, 1983).

CNBr-activated Sepharose™ 4B gel was rehydrated according to the manufacturer's instructions [Appendices 1:4 & 3]. Following rehydration the gel was filtered and immediately added to a solution containing the dialysed TPO protein which was diluted in approximately 3 ml of coupling buffer, pH 8.3 [Appendix 2:4]. This stage should be completed without delay since reactive groups on the gel hydrolyse at the coupling pH.

The crude TPO sample (500 µl, providing a protein content of 5 mg of protein), which had been dialysed overnight in bicarbonate buffer [Appendix 2:4], was added to the CNBr gel and roller mixed for 2 hours at room temperature, checking that all the gel was in suspension. (For protein ligands a concentration of 5-10 mg of protein per ml of CNBr Gel was recommended).

The suspension was then spun down and the protein content of the supernatant estimated spectrophotometrically at 280 nm. The percentage uptake of protein (by the gel) from the initial dialysate was calculated to assess the efficiency of the labelling procedure.

The gel was then transferred to 1M ethanolamine, pH 8.0, and the contents were roller-mixed for a further 2 hours at room temperature. The 1M ethanolamine acts as a blocking agent and helps minimise non-specific adsorption to immunoadsorbents.

The next stage involved washing the gel 5 times in alternating high and low pH buffers, (i.e. between coupling buffer, pH 8.0 and acetate buffer, pH 4.0) [Appendix 2:4]. This procedure ensures that no free ligand remains ionically

bound to the immobilised ligand. After the final wash, the gel was resuspended in 5 mls of TBS Azide, pH 7.4 [Appendix 2:4], and stored at 4°C prior to use.

2.7.6 (ii) Purification of Acrid-IgG Label Using Affinity Chromatography

The TPO gel was transferred to a suitable support system (e.g. 5 ml plastic pipette tip), with the end being plugged with cotton wool. The gel was washed with approximately 4 times the bed-volume (i.e. 10 ml), with elution buffer (TBS Azide, pH 7.4, 0.1% BSA [Appendix 2:4]. The column was not allowed to dry.

The 1 ml of Acrid-IgG label (labelled as described in 2.6.2 & 2.7.3), was carefully transferred to the top of the gel. The column was eluted slowly with 15 ml of elution buffer to allow adequate time for the Acrid-IgG label to specifically bind to the TPO gel. The eluate was collected in fifteen 1 ml fractions and 10 µl of each fraction was diluted in 1 ml of TBS /1% BSA and the RLUs of a 10 µl aliquot of this dilution was measured as before.

The non-specifically bound substances were eluted in the first 3 fractions with TBS / Azide, pH 7.4 [see Fig 42 for the elution profile]. The purified Acrid-IgG label was finally eluted in 3M sodium thiocyanate solution in fractions 2 and 3 [indicated as fractions 16 and 17 in elution profile: see Fig 43]. Previous fractions 1 to 14 represented the failed attempt to elute the label in 2M NaCl. The fractions containing the Acrid-IgG label were dialysed (in visking tubing, Size 1-8) overnight against TBS/Azide, pH 7.4 and the dialysate of each fraction stored frozen until use.

2.7.7 Assay Using the Further Purified Acrid-IgG Label

Standards of the 'in-house' MicAb/TPOAb preparation were diluted to give concentrations ranging from 3.125 to 100 kIU/L [see 2.2.2] and appropriately diluted quality control [see 2.3] were assayed [see 2.7.5] using the Biotin-TPO label (100 µl), (both diluted 1:100 and 1:1000 using TBS / 1% sheep

serum); 100 µl of the further-purified Acrid-IgG label (diluted 1:100 with PBS / 1% sheep serum) and 10 µl of the Dynabeads®. A range of 'L4' control dilutions from 1:10 to 1:10,000 were also assayed, with the same Biotin-TPO and Acrid-TPO label dilutions.

2.8 Method 3 (System 3)

Solid-Phase, Non- Competitive Immunoassay Using Sheep Anti-Human IgG Labelled with Acridinium Ester (Coated-Tube Assay)

2.8.1 Principle of Assay

This was a solid-phase, heterogeneous (sandwich) assay system, in which the TPOAb in the sample reacted with the TPO antigen coated on the tube, and then binds to the sheep anti-human IgG labelled with acridinium ester (Acrid-Anti-IgG) to form a labelled complex. This was a non-competitive assay system resulting in a direct correlation between the RLUs attached to the tube and the concentration of TPO autoantibodies in the sample, i.e. the sample antibody concentration is proportional to the final RLUs [see Fig 14].

2.8.2 Procedure for Labelling Sheep Anti-Human IgG with Acridinium Ester

A preparation of sheep anti-human IgG (Sheep Anti-Human IgG [Gamma Chain] obtained from Serotec Ltd.,) (with an approximate concentration of 0.5 - 1 mg/ml), was used. A 200 µl aliquot of the diluted sheep anti-human IgG (40 µl of the anti-serum diluted with 180 µl of phosphate buffer, pH 7.4), was added to a vial of acridinium ester (5 µg) and the labelling and purification procedures carried out as described in 2.6.2, 2.6.4 & 2.7.3).

2.8.3 Assay Using Tubes Coated with TPO and Sheep Anti-Human IgG Labelled with Acridinium Ester

Sarstedt tubes were coated with 100 µl of TPO (solubilised with 1% deoxycholic acid and diluted with coating buffer, pH 9.3 [Appendix 1:5] to give a concentration of 1 µg TPO/ml. The tubes were incubated overnight at 4°C, the supernatant decanted and the tubes drained thoroughly. The tubes were washed four times using the ELISA wash [Appendix 1:5], and thoroughly drained. To duplicate coated tubes, 100 µl of each of the standards [diluted as for Method 3 (Systems 1 & 2) and ELISA], and quality

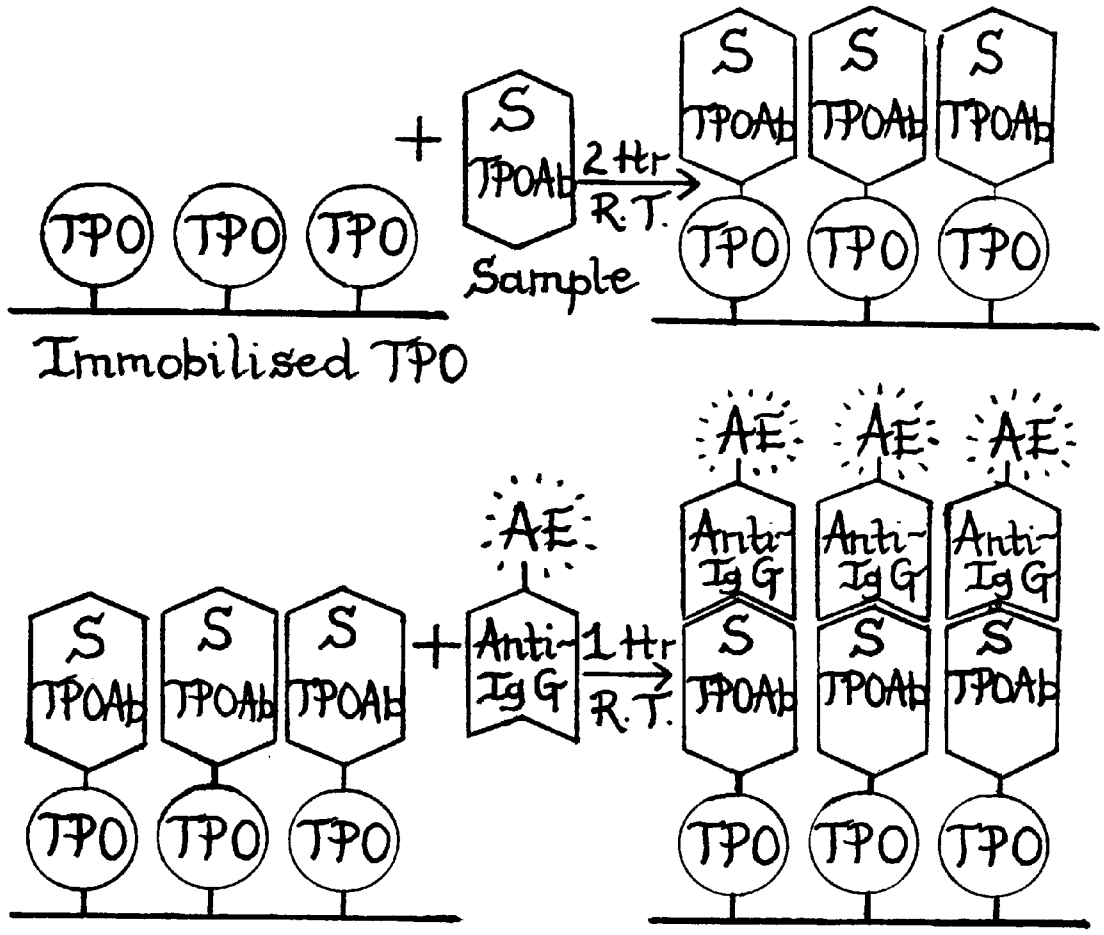


Figure 14 Diagrammatic Representation of Coated-Tube Assay (System 3).

Microsomal TPO immobilised on plastic support, reacted with sample thyroid peroxidase autoantibody (TPOAb-S), which then reacted with sheep anti-human IgG labelled with AE. The final RLUs attached to the tube provided a direct relationship with TPOAb-S.

control and samples (diluted 1:100 with PBS diluent, pH 7.4) [Appendix 1:5], were added and incubated at room temperature for 2 hours. The tubes were decanted and again washed four times. The Acrid-Anti-IgG label was diluted 1:1000 with PBS, pH 7 with 1% sheep serum and added to the tubes which were incubated at room temperature for a further hour. The tubes were finally decanted and washed a further four times before measuring the RLUs attached to each tube as described previously.

Assays were set up using different dilutions of the Acrid-Anti-IgG label and different incubation periods. Precision and comparison studies were carried out, together with experiments to determine the analytical sensitivity and specificity and also the 'high-dose hook' effect [see 3.5] .

2.8.4 Estimation of the Analytical Sensitivity of the Coated-Tube Assay

Blank samples consisting of PBS, pH 7.4, were assayed (as described in 2.8.2) and the analytical sensitivity was calculated. The definition and subsequent calculation of analytical sensitivity used was as defined by Bayer/Chiron Diagnostics in their Chemiluminescent automated assays, i.e. the concentration of analyte that corresponds to the RLUs that are two standard deviations greater than the mean RLUs of 'x' replicate determinations (20 replicates used in this study).

2.8.5 Statistical Analyses

The logarithmically converted data for the standard curves for Method 3 (CIA): Systems 1-3, were graphically plotted, and the correlation coefficient (r) for each assay was derived, (which indicated the closeness of the transformed data to a linear response). The closer (r) is to 1.0 the closer the 'fit' of data to a straight line.

The logarithmically converted results of the coated-tube assay and the ELISA were statistically compared using the 'least squares' method of regressional analysis (using Microsoft® Excel: Version 5). The 'Deming Plot' and 'Passing & Bablok' Regression Models were also applied (using 'Analyse

It': Version 1.28). The results of the agglutination assay had to be 'rank-transformed' for statistical analysis, and a semi-log regression plot was used to compare this method with the coated-tube assay and the ELISA [see 3.5 for results]. The non-parametric 'Wilcoxon Mann-Whitney 'U' Test' was also applied for comparing two groups of results (using Minitab: Version 9).

CHAPTER 3

RESULTS

Chapter 3: Results

3.1 Method 1

Immunoradiometric Assay (IRMA) Using TPO Labelled with [^{125}I] and Magnetic Particles Coupled to Protein A

A limited study only was carried out for the IRMA [see 2.4], with the primary aim being to assess the viability of the magnetic particles coupled to Protein A, which were to be subsequently incorporated as the separation phase in an enzyme-immunometric assay [see 2.5].

The percentage uptake of Protein A by the magnetic particles (estimated using differential protein measurements of the supernatants before and after coupling) [see Appendix 2:2 for further details], achieved an average uptake of 94% on separate preparations, which indicated a successful and reproducible coupling procedure.

3.1.1 Experiments to Determine the Percentage Uptake of IgG by the Protein A Suspension

Experiment 1

Aliquots (100 µl) of the Protein A Suspension (i.e. neat and diluted in a range from 2-fold to 20-fold with 0.1M phoshate buffer, pH 7.4) [Appendix 1:1], were incubated with 100 µl of a serum diluted in Tris HCl, pH 7.5 [Appendix 1:1] to give a final concentration of 0.98 g/L immunoglobulin G (IgG) per tube. The tubes were incubated overnight (with the experiment being carried out at room temperature and at 37°C). The IgG present in the separated supernatant was estimated using a turbidimetric method (for the routine determination of immunoglobulins), in which the immunoglobulin G was mixed with its specific anti-IgG anti-serum (obtained from Dako Ltd.,) and the resulting turbidity measured spectrophotometrically on the Cobas Fara II centrifugal analyser at 340 nm [see Appendix 2:3 for a description of the method].

The uptake of immunoglobulin G (IgG) by the neat Protein A suspension (with overnight incubation) was 0.56 g/l (57%) at room temperature and 0.49 g/l (49%) at 37°C, (an average of 53% of the quoted uptake of 9 mg of IgG per 1 mg of Protein A). The greater uptake of IgG was evident at room temperature for all Protein A concentrations [see Figs 15 & 16].

Figure 15
Uptake of IgG by the Protein A Suspension
(Overnight Incubation: At Room Temperature)

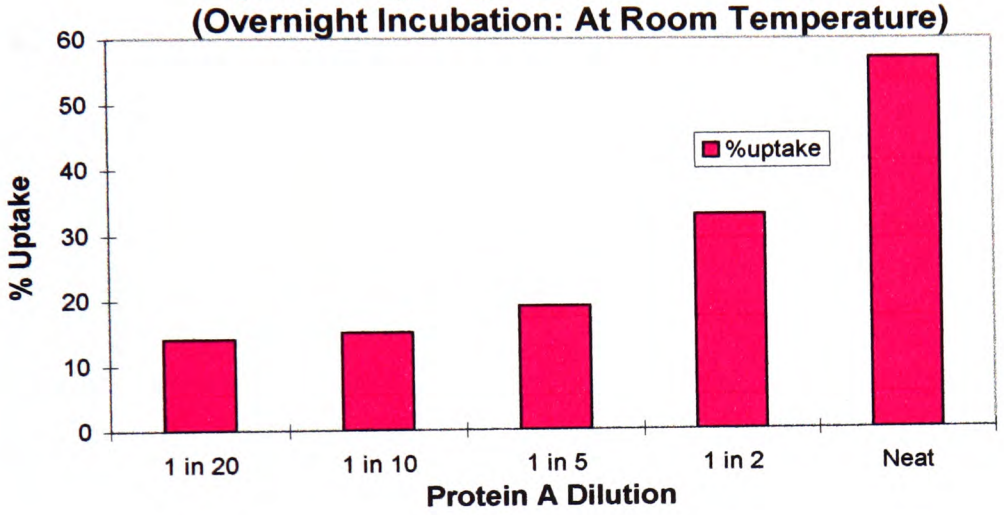
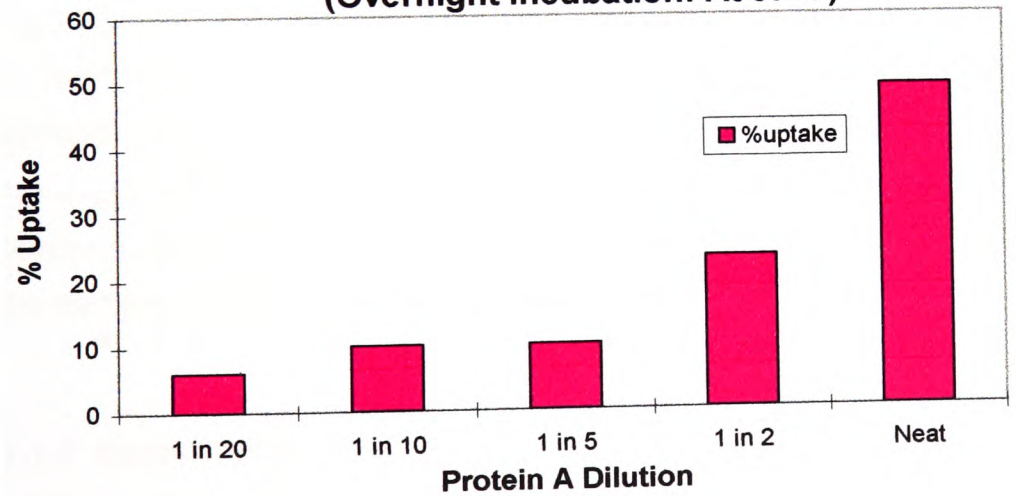


Figure 16
Uptake of IgG by the Protein A Suspension
(Overnight Incubation: At 37°C)



Figures 15 & 16: The uptake of IgG with different concentrations of the Protein A suspension at different temperatures.

Experiment 2

A further experiment to investigate the percentage uptake of the Protein A suspension, involved the overnight incubation, at room temperature of different dilutions of serum IgG (50-fold, 100-fold and 200-fold) with various dilution ratios of the Protein A suspension (1:10, 1:2.5 and neat).

Figure 17
Uptake of Different Concentrations of the Protein A Suspension with Different IgG Sample Concentrations

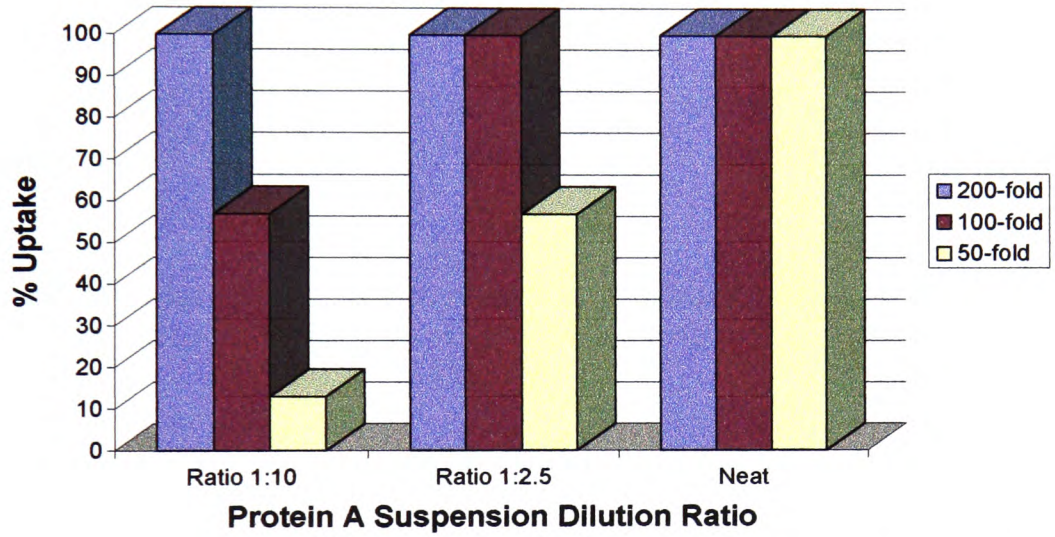


Fig 17 shows that the neat Protein A suspension was completely successful in removing all the IgG from all of the sample dilutions. The magnetic particles diluted in a ratio 1:10, were less effective and only succeeded in removing all the IgG when the sample was diluted 1:200. The magnetic particles diluted in a ratio 1:2.5 exhibited complete uptake of the IgG when the sample dilution was 1:100 or 1:200.

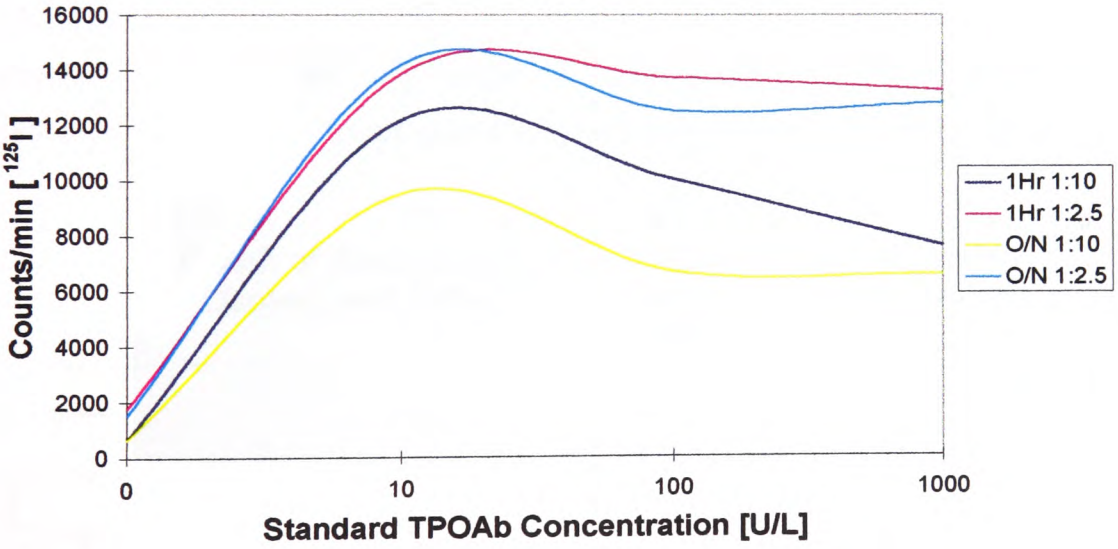
3.1.2 Experiments Varying Incubation Periods

Assays were carried out varying the initial (i.e. 1 hour and overnight) and the final (i.e. 1 hour and 30 minutes) incubation periods (all at room temperature), with various dilutions of the Protein A suspension (i.e. 1:10, 1:5 and 1:2.5). However, varying either of the incubation periods did not significantly affect assay results.

The first incubation period was compared overnight and for 1 hour, with the final incubation period of 1 hour (using the Protein A suspension diluted 1:10 and 1:2.5.

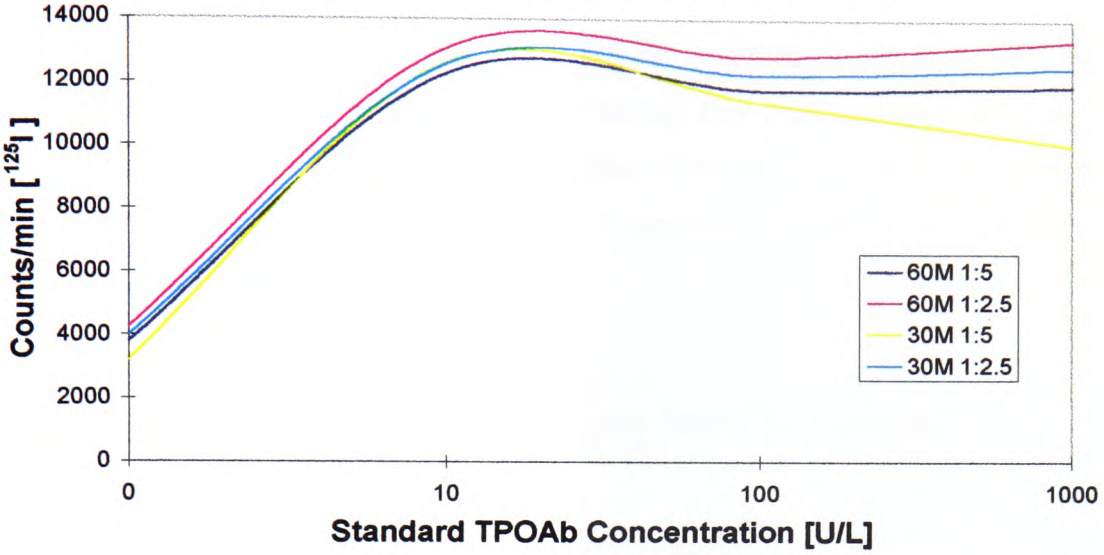
The assays with the initial incubation period of 1 hour, exhibited a progressive decrease in counts at the upper TPOAb standard concentrations, with the decrease being not as pronounced with the overnight incubation, which generally exhibited a levelling off in the final response after an initial decline. The Protein A suspension diluted 1:10, demonstrated the 'high-dose hook' effect, (in which there was the paradoxical decrease in radioactive counts at very high serum autoantibody concentrations), which was not as pronounced using the suspension diluted 1:2.5 [see Fig 18].

Figure 18
Standard Curves Using Different Concentrations of Protein A Suspension with Different Initial Incubation Periods



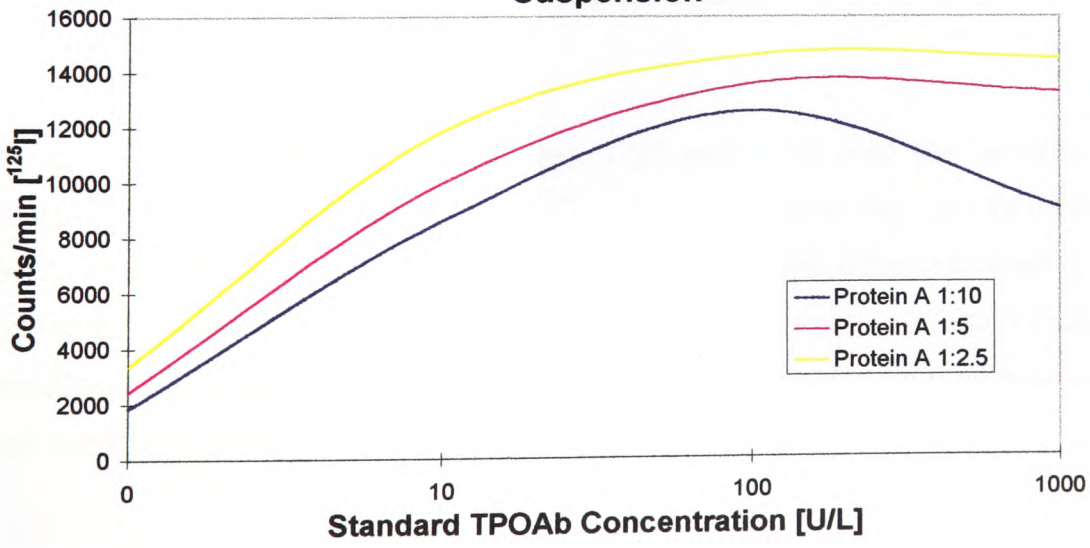
Similarly, the assay was carried out with an initial incubation period of 1 hour and the final incubation of both 1 hour and 30 minutes (using the Protein A suspension diluted in a ratio 1:5 and 1:2.5). All incubations were at room temperature. A slight final decline in response was evident with the Protein A suspension diluted in a ratio 1:5 with the final incubation of 30 minutes [see Fig 19].

Figure 19
Standard Curves with Final Incubation Periods of 30 Minutes (30M) and 1 Hour (60M): Using Different Concentrations of Protein A Suspension



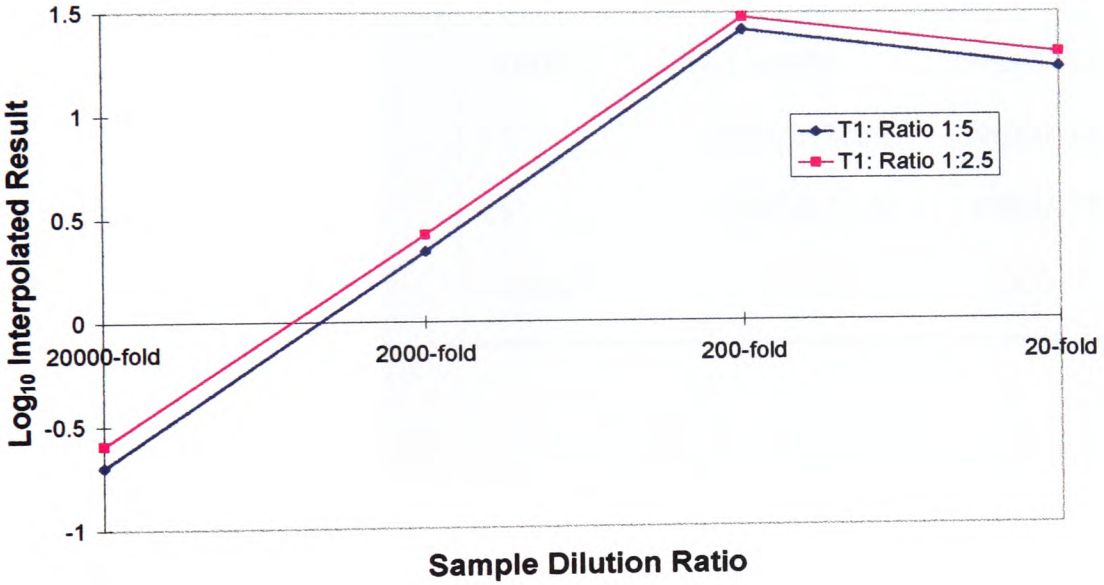
The final assay conditions consisted of both incubation periods of 1 hour at room temperature, with an optimum Protein A suspension dilution ratio of 1:5. The graphs shown in Figs 18 & 19 indicated that 10 U/L was the effective top of the working range. In future assays the stock standard was further diluted 20-fold to provide a working range [see 2.2.1 & Fig 20].

Figure 20
Graph of Assays (Using the TPOAb Standard Further Diluted) and Different Concentrations of Protein A Suspension



A sample (T1) containing a high thyroid peroxidase autoantibody (TPOAb) activity (diluted from 20-fold to 2000-fold) was assayed [see 2.4.5], and the results obtained were read off the standard curve. The results indicated an excellent recovery, and the logarithmic conversion of the interpolated data produced an initial linear response up to a dilution of 200-fold, with a subsequent decrease in counts representing the manifestation of the 'high-dose hook' effect. The experiment was carried out using the magnetic particles diluted in a ratio 1:2.5 and 1:5 and both gave a similar response [see Fig 21].

Figure 21
Graph of Results of Recovery Study for Sample T1



A set of 10 samples were all diluted 1:20 and 1:50 and the results were interpolated from a standard curve and compared with the results obtained by indirect agglutination (InAg) [see 2.4.6]. The limited data indicated a good correlation between the two methodologies. Further sample dilution would be required to provide absolute values for those analyses which exceeded the top standard [see Table 1].

Table 1 Results of IRMA and Indirect Agglutination (InAg) Comparison

SAMPLE No.	IRMA Sample1:20	IRMA Sample1:50	InAg Microsomal	InAg Thyroglobulin
1.	<1	<1	1:400	1:12,000
2.	<1	<1	NEGATIVE	NEGATIVE
3.	<1	<1	NEGATIVE	NEGATIVE
4.	>1000	>1000	1:25,000	1:200
5.	6	9	NEGATIVE	NEGATIVE
6.	>1000	>1000	1:25,000	NEGATIVE
7.	>1000	>1000	1:6,400	NEGATIVE
8.	<1	<1	NEGATIVE	NEGATIVE
9.	<1	<1	NEGATIVE	NEGATIVE
10.	>1000	>1000	1:25,000	1:800

3.2 Method 2

Enzyme-immunometric Assay Using TPO Labelled with Horseradish peroxidase (HRP) and Magnetic Particles Coupled to Protein A

3.2.1 Experiments to Determine the Enzyme Activity of the HRP Pre- and Post-Coupling with TPO

The initial HRP dilution and the HRP-TPO label were both diluted 1 in 1000, and 1 in 10,000 with double-distilled water. A 10 µl aliquot of each of the dilutions were added to 1 ml of freshly prepared o-phenylenediamine dihydrochloride (OPD) substrate reagent [Appendix 1:2], and the enzyme activity was monitored spectrophotometrically, by measuring the rate of change in absorbance at 492 nm. A 22% retention only of the initial HRP activity was demonstrated in the post-coupling HRP-TPO preparation.

A series of dilutions of the HRP-TPO label (250-fold, 500-fold and 1000-fold), were prepared using 0.5M PBS, pH 7.4, and 100 µl each of the dilutions were incubated with 1 ml of OPD substrate reagent [Appendix 1:2] for 30 minutes in the dark, at room temperature. The reaction was stopped by the addition of 50 µl of 10M NaOH and the absorbance of the supernatants was measured at 492 nm and the results, which were graphically represented demonstrated a linear response [see Fig 22].

The enzyme-immunometric assay (described in 2.5.4) produced a 'blanket' response with similar absorbances at 492 nm for all standard TPOAb concentrations (the results were confirmed by repeat analysis with the repreparation of all assay components) [see Fig 23].

The OPD substrate was replaced with 1ml of 5 mM potassium iodide (KI) and the reaction stopped by the addition of 15 µl of 30% hydrogen peroxide (H_2O_2) and the optical density was measured at 350 nm, but again a similar 'blanket' response was produced for all the standard preparations.

Figure 22
Graph of HRP-TPO Label Dilutions and Reaction with OPD Substrate

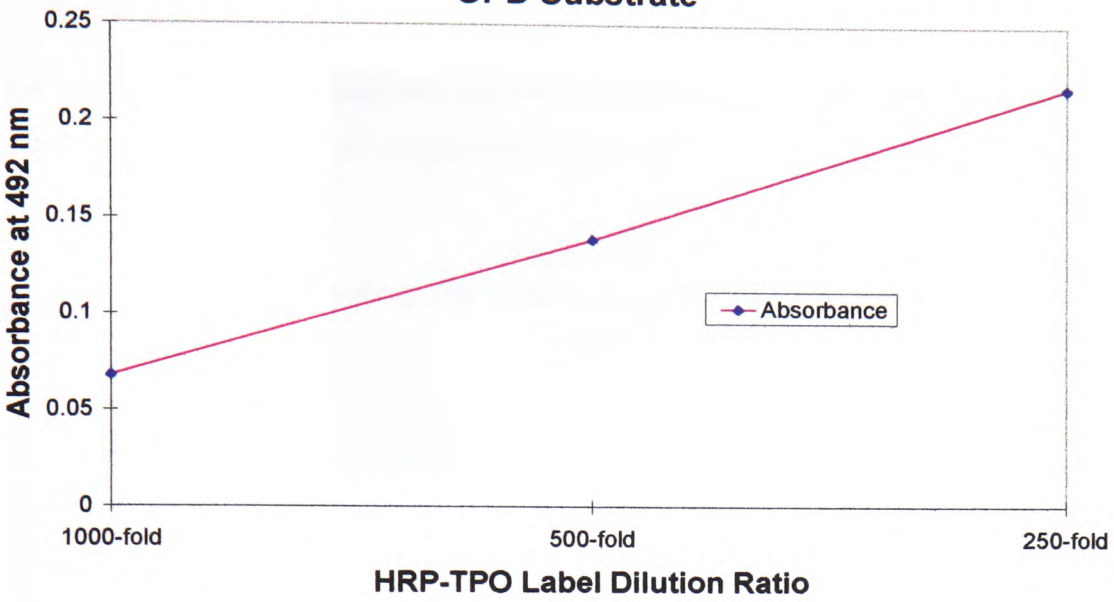
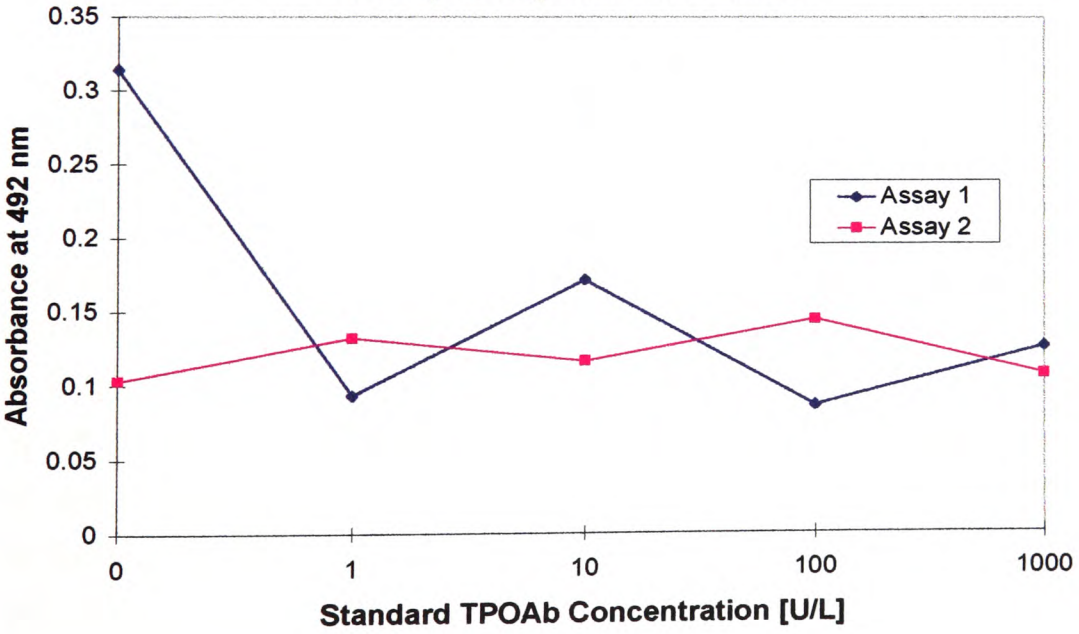


Figure 23
Graph of Standard Curves for the Enzyme-Immunometric Assay Using HRP-TPO Label

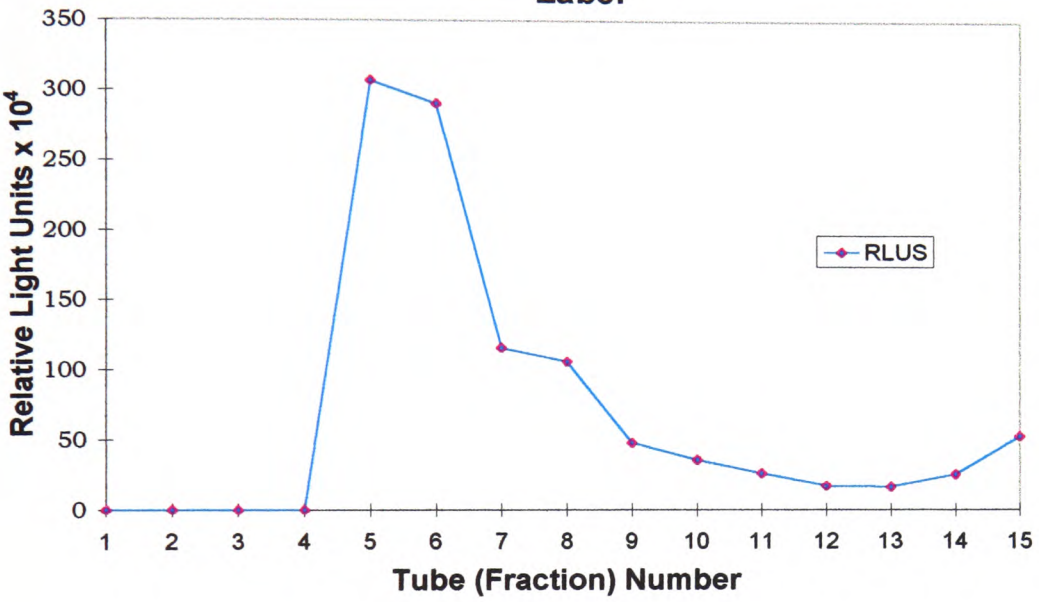


3.3 Method 3 (System 1)

Chemiluminometric Assay Using TPO Labelled With Acridinium Ester and Magnetic Particles Coupled to Anti-Human IgG

The Acrid-TPO label (labelled as described in 2.6.2) was consistently eluted in fractions 5 and 6 on all preparations [see Fig 24].

Figure 24
Graph Showing the Elution Profile of the Acrid-TPO Label

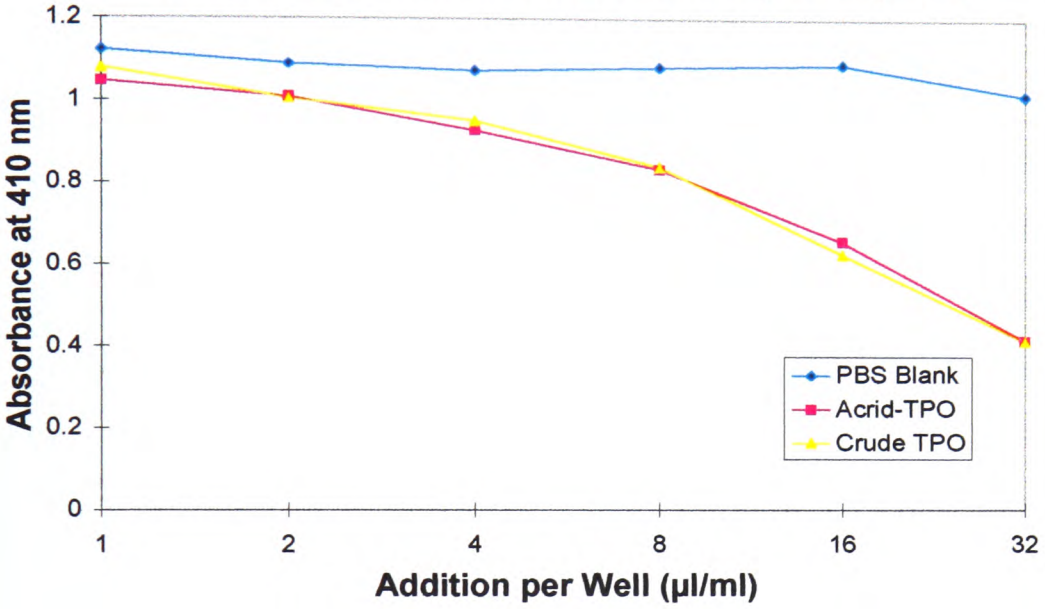


3.3.1 Experiment to Assess Whether the Antigenic Properties of TPO are Modified After Coupling To Acridinium Ester

An assay was set up using the ELISA system (Groves *et al.*, 1990) to compare the antigenicity of the TPO labelled with AE (Acrid-TPO) with that of the original crude microsomal preparation. Increasing concentrations of Acrid-TPO label (from 0 to 160 µl/ml) were added in a final volume of 100 µl per well, across an ELISA plate [coated with 1 µg/ml TPO (solubilised with 1% deoxycholic acid)]. The label was diluted in a sample containing a high TPOAb titre (diluted 1:100 in PBS, pH 7.4). The crude TPO (diluted 1:10) and PBS diluent (as the blank) were similarly diluted and assayed and their response compared with that of the Acrid-TPO label.

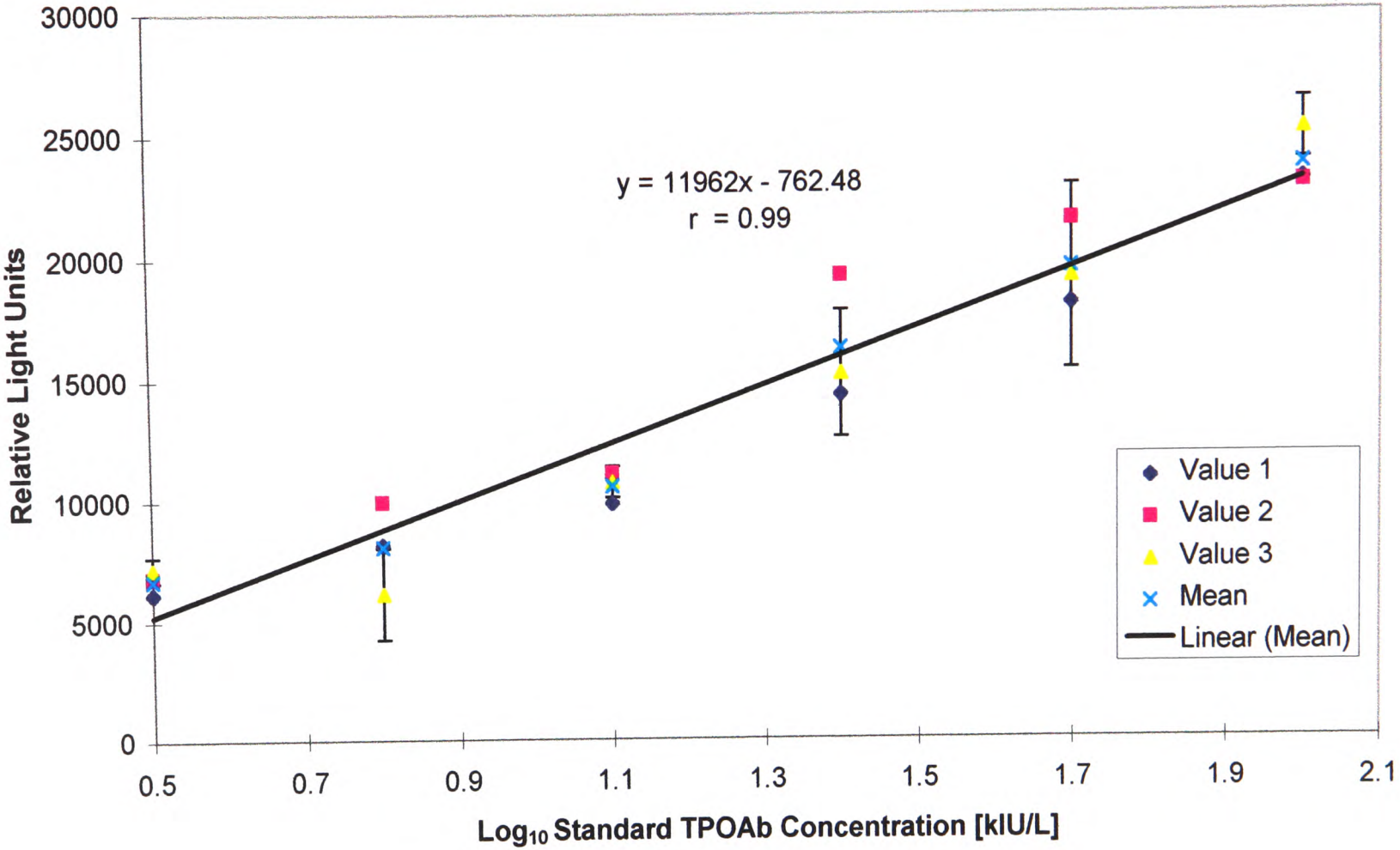
The experiment indicated that no apparent change or loss of antigenicity of the TPO had occurred upon labelling with acridinium ester (AE) as compared with the unlabelled TPO [see Fig 25].

Figure 25
Graph Comparing the Antigenic Response of the Acrid-TPO Label with Unlabelled (Crude) TPO



The results of an initial assay using a label prepared with 5.5 µg of AE and a solubilised preparation of TPO produced a correlation coefficient (r) of 0.99 upon regression curve analysis (with the mean of triplicate analyses used). A lack of precision was demonstrated with the overlapping of consecutive standard data points, with the error bars being defined as 1 standard deviation (S.D.) of the mean. A plot of relative light units (RLU) instead of Log_{10} RLU (on y-axis) is shown to illustrate the assay imprecision [see Fig 26]. In addition, only an uptake of 0.5% of AE measured in RLUs was exhibited by the top standard which did not augur well for adequate assay sensitivity.

Figure 26
Graph of Relative Light Units versus Log_{10} Standard TPOAb Concentration for the Chemiluminometric Assay [Method 3 (System 1)]



3.3.2 Experiments Varying Acrid-TPO Label Dilutions

Varying the ratios of AE to TPO protein in the preparation of the labels [see 2.6.3] produced no improvement in results. Inferior results were produced for all labels [i.e. prepared with 5 µg (100%), 2.5 µg (50%), 0.5 µg (10%) and 0.05 µg (1%) of AE], as compared with the standard curve using 5.5 µg, with correlation coefficients (r) values of between 0.88 and 0.97 produced on regression curve analysis of the mean of duplicate analyses. Variable precision was still evident over the standard data points [see Figs 27-30: error bars defined as 1 S.D.].

A greater uptake of AE for each standard was produced with increasing amounts of AE in the label. The greatest percentage uptake of AE by the top standard was achieved with the label containing 1% AE, but then only with an uptake of 1.1%. In addition, a poor curve response was produced with the 1% label [see Fig 30], with no apparent uptake of AE and no discrimination between consecutive standards.

A deterioration in assay performance was demonstrated with labels prepared with crude TPO as compared with TPO solubilised with 1% deoxycholic acid. A label prepared with 5 µg (100%) of AE produced a correlation coefficient (r) of 0.96 (upon regression analysis) for the crude TPO as opposed to 0.98 for the solubilised TPO. However, labels prepared with 0.5 µg of AE produced similar correlation coefficient (r) values of 0.94 and 0.96 for the solubilised and crude TPO respectively. A greater uptake of AE was exhibited across the standard curve for the label prepared with the solubilised TPO [see Fig 31].

Figure 27
Graph of Assay Using Acrid-TPO Label Prepared with 5 µg (100%) of Acridinium Ester

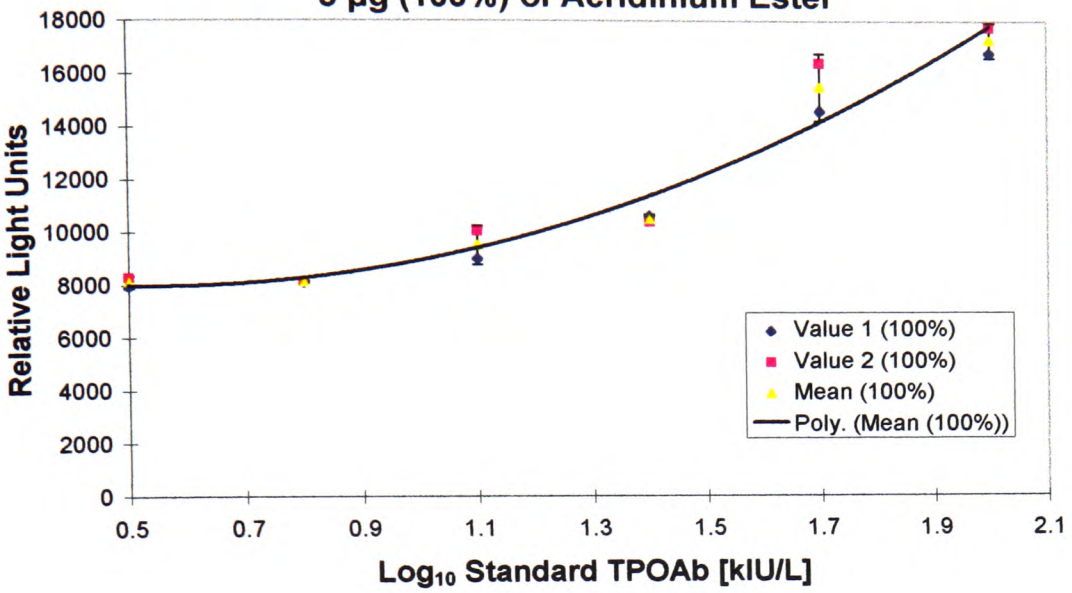


Figure 28
Graph of Assay Using Acrid-TPO Label Prepared with 2.5 µg (50%) of Acridinium Ester

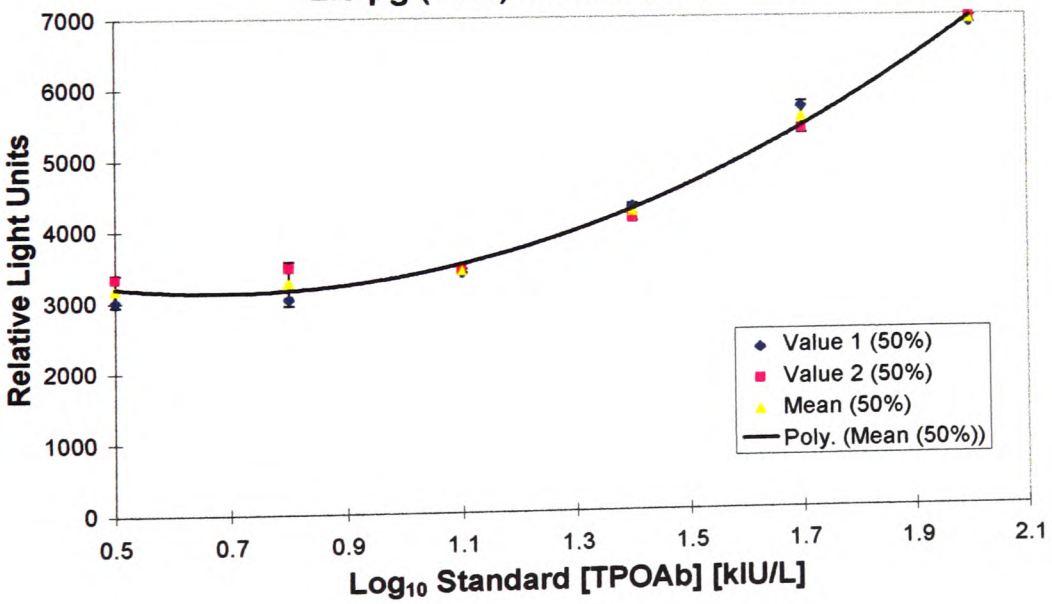


Figure 29
Graph of Assay Using Acrid-TPO Label Prepared with 0.5 µg (10%) of Acridinium Ester

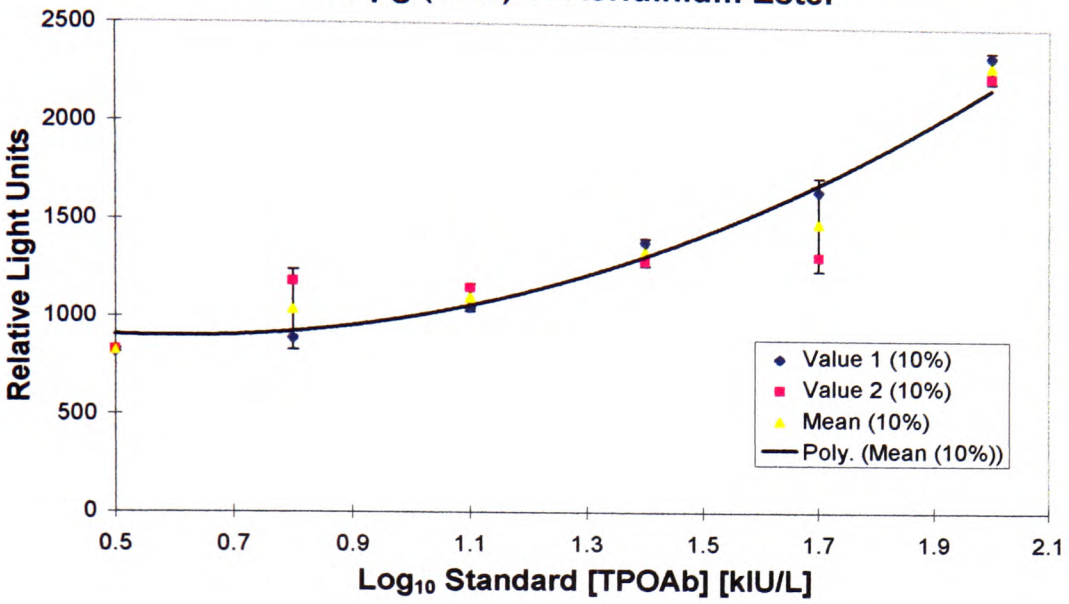


Figure 30
Graph of Assay Using Acrid-TPO Label Prepared with 0.05 µg (1%) of Acridinium Ester

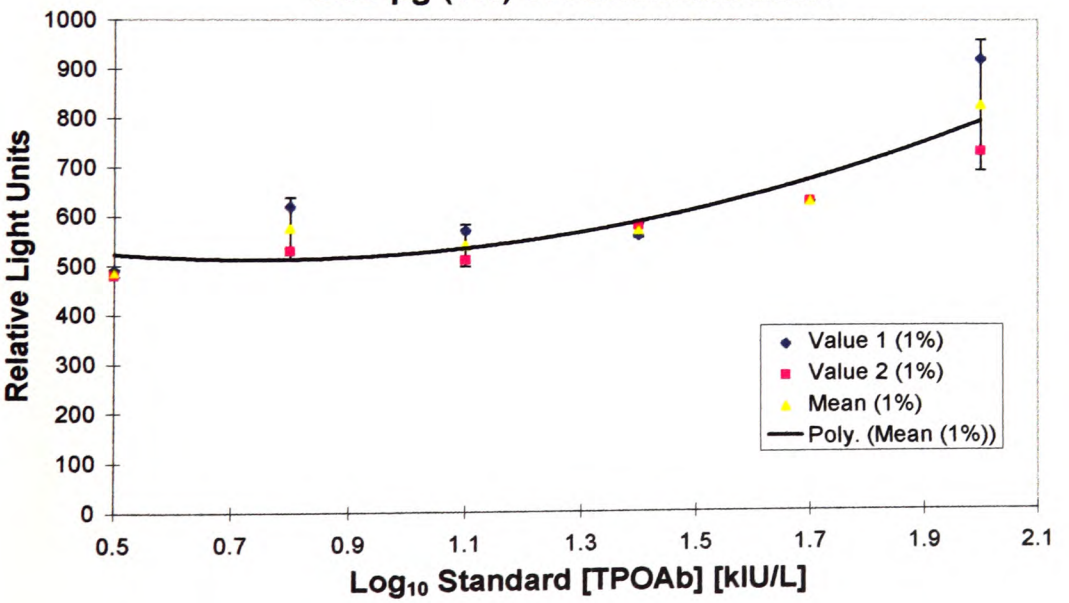
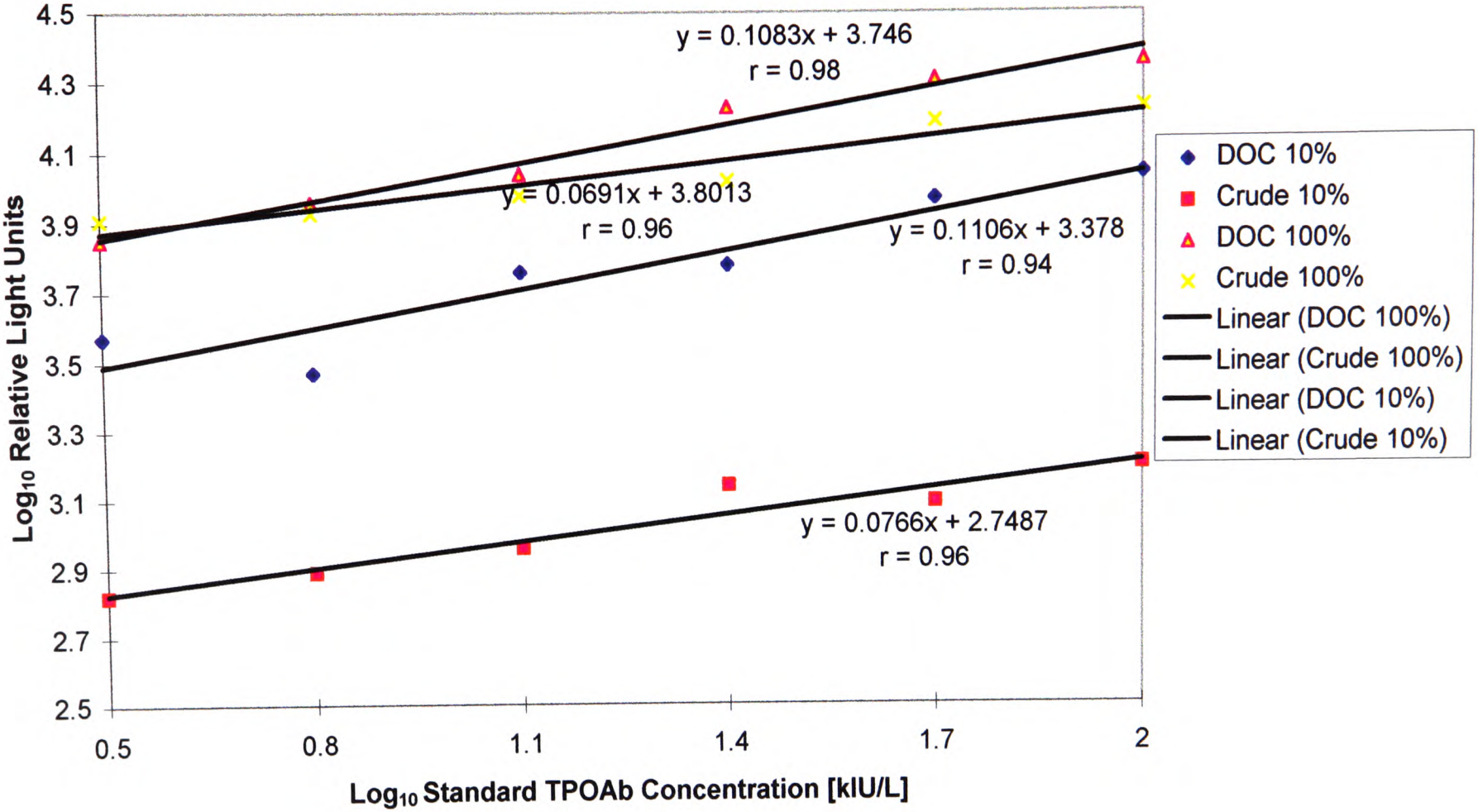
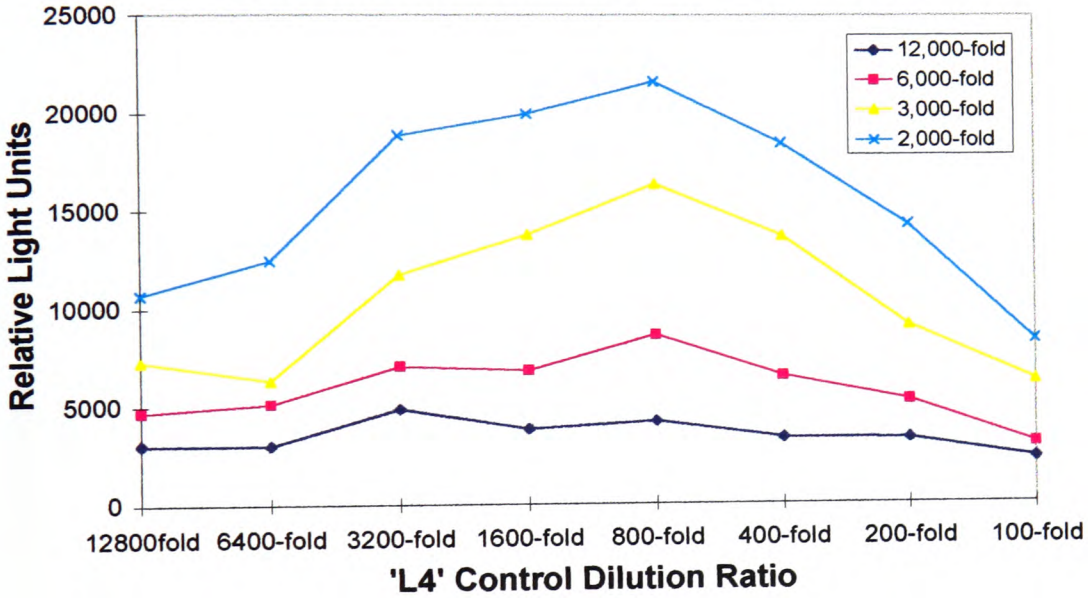


Figure 31
Graph Showing the Comparison of Acrid-TPO Label Prepared from DOC-Solubilised TPO and Crude TPO : Method 3 (System 1)



A series of dilutions of the high 'L4' control (100-fold to 12800-fold, diluted in sample diluent), were assayed (as described in 2.6.5), using different dilution ratios of the Acrid-TPO label (prepared using 5.5 µg of AE). The label was diluted (in label diluent) to give the ratios 12,000-fold, 6,000-fold, 3,000-fold and 2,000-fold and assayed using the MAb-Anti-IgG diluted 1:20 [see Fig 32].

Figure 32
Graph of 'L4' Control Dilutions Using Different Concentrations of the Acrid-TPO Label



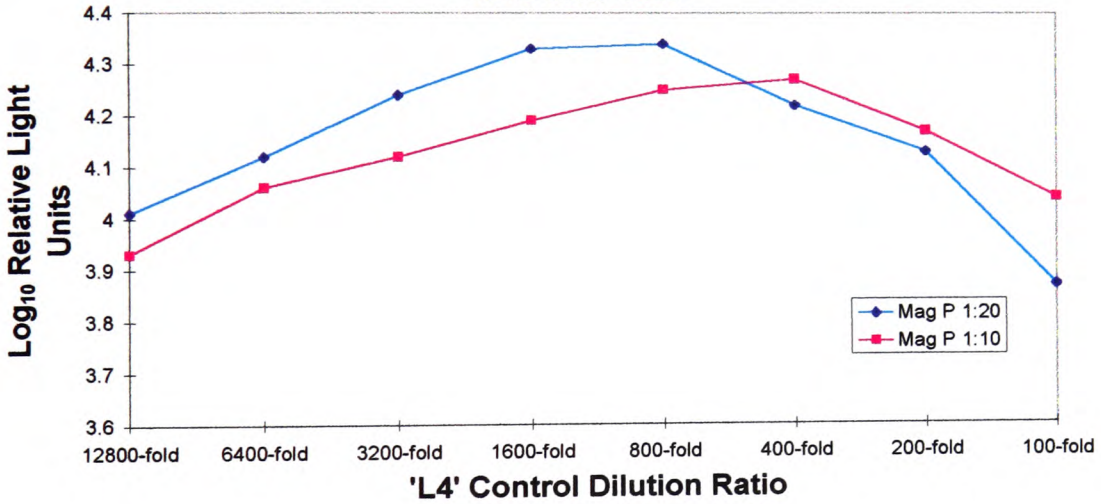
Increased imprecision was demonstrated in the duplicate analyses with increasing Acrid-TPO label concentration, with very little discrimination between consecutive 'L4' dilutions using the Acrid-TPO diluted 12,000-fold. Although imprecision obscured the results, there was an indication of the 'high-dose hook' effect for all the other Acrid-TPO label dilutions at an 800-fold dilution of the 'L4' control with the MAb-Anti-IgG diluted 1:20 [see Fig 32].

3.3.3 Experiments to Investigate the Efficacy of the MAb-Anti-IgG

Standard curves were set up using double the concentration of magnetic antibody (i.e. stock diluted 1:10) and the Acrid-TPO label prepared with 5.5 µg of AE, demonstrated no improvement in assay results (data not shown).

The 'L4' control dilutions were also assayed using the Acrid-TPO label (prepared with 5.5 µg) and the MAb-Anti-IgG diluted both 1:10 and 1:20. The results indicated the 'high-dose hook' effect at an 'L4' dilution of 800-fold with the magnetic antibody diluted 1:20, and at a dilution of 400-fold for the particles diluted 1:10 [see Fig 33].

Figure 33
Further Investigation of the 'High-dose Hook' Effect Using the Magnetic Particles (MAb-Anti-IgG) Diluted 1:10 and 1:20



Another experiment involved the dilution of the high 'L4' control both 400-fold and 800-fold in both sample diluent and in a TPOAb positive sample and assayed (as described in 2.6.6) with MAb-Anti-IgG diluted both 1:20 and 1:10.

In the first set of tubes, Tubes 1 and 3 consisted of the high TPOAb positive 'L4' control diluted 800-fold [(with a TPOAb negative sample (determined via an ELISA) [Appendix 2:7] (which had also been diluted 800-fold with assay diluent)]. Tubes 1 and 3 were assayed with the MAb-Anti-IgG diluted 1:20

and 1:10 respectively. Tubes 2 and 4 consisted of the 'L4' control diluted 400-fold with assay diluent, with the MAb-Anti-IgG diluted 1:20 and 1:10 respectively [see Table 2 (i)].

In the second set of tubes, Tubes 2 and 4 were as the first set, but Tubes 1 and 3 consisted of the 'L4' control diluted 800-fold with a TPOAb negative sample (which had been pre-diluted 400-fold with assay diluent), with the MAb-Anti-IgG diluted 1:20 and 1:10 respectively [see Table 2 (ii)]. The latter dilution resulted in a greater final sample protein concentration.

Table 2

Results of Experiment to Further Determine the Efficacy of the Magnetic Antibody (MAb-Anti-IgG)

Tube 1	Tube 2	Tube 3	Tube 4
'L4' Control (800-fold) (mix)	**'L4' Control (400-fold)**	**'L4' Control (800-fold) (mix)**	**'L4' Control (400-fold)**
[MAb 1:20]	**[MAb 1:20]**	**[MAb 1:10]**	**[MAb 1:10]**
(i) 14930 RLU	8380 RLU	13340 RLU	17570 RLU
(ii) 12240 RLU	9560 RLU	12290 RLU	15440 RLU

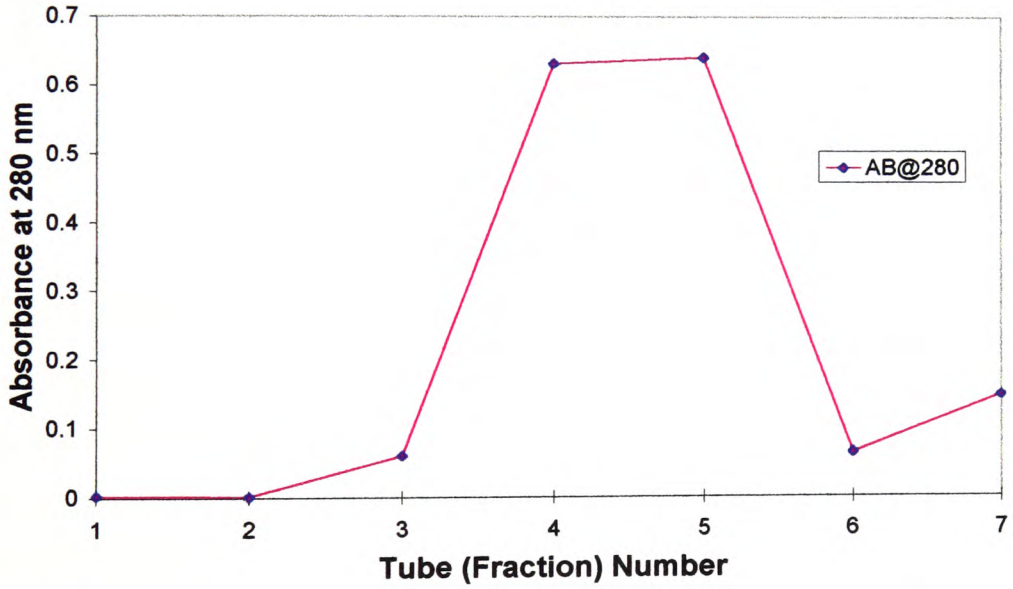
A similar response was produced for the 'L4' control diluted 800-fold for both magnetic antibody concentrations. However, the magnetic antibody diluted 1:20 appeared to be rate-limiting for the 'L4' control diluted 400-fold, with RLUs of 8,380 as compared to 17,570 RLUs produced for the 1:10 dilution ratio, thus, demonstrating the 'high-dose hook' effect with low RLUs for a high TPOAb titre [see Table 2 (i)]. Similar results were produced with the dilutions which incorporated different sample protein dilution ratios of a TPOAb negative sample [see Table 2 (ii)].

3.4 Method 3 (System 2)

Competitive Immunoassay Using TPO Labelled with Biotin, Anti-Human TPO Labelled with Acridinium Ester and Magnetic Beads Coupled to Streptavidin

The preparation of the Biotin-TPO label (using the ECL Protein Biotinylation Module) [see 2.7.2 (i)] appeared to be successful with a >50% uptake of the solubilised TPO (estimated by spectrophotometric measurement at 280 nm). The biotinylated TPO label (Biotin-TPO) purified from the 'free biotin' via gel filtration using Sephadex™ Gel 50M [see 2.7.2 (ii)], was eluted within the first five fractions, which agreed with ECL's protocol [see Fig 34].

Figure 34
Graph Showing the Elution Profile of the Biotin-TPO Label



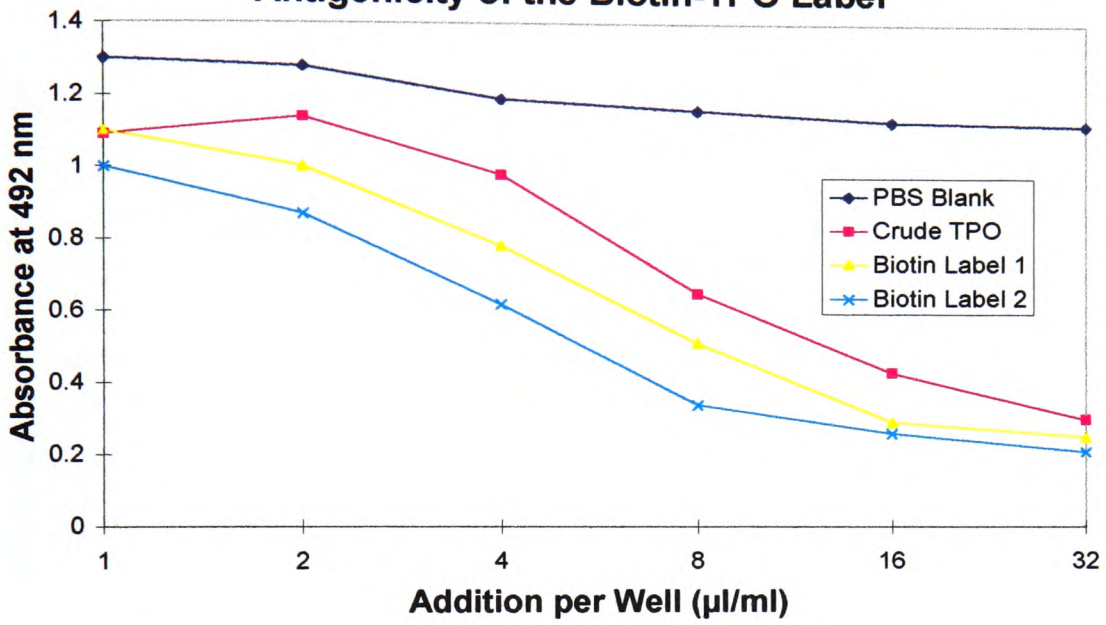
3.4.1 Experiments to Investigate the Antigenicity of the Biotin-TPO Label

Experiment 1

The antigenicity of the Biotin-TPO label was similarly investigated as described for the Acrid-TPO label [see 3.3.1], with increasing amounts of label (diluted in a high TPOAb sample) being added across an ELISA plate and comparing its response with with an equivalent concentration of the

crude microsomes and PBS diluent (as blank). The Biotin-TPO produced a similar response (with a decrease in absorbance with increasing TPO added) as compared with the crude unlabelled TPO, which indicated no apparent loss of antigenicity of the TPO upon labelling [see Fig 35].

Figure 35
Graph of Experiment to Investigate Any Change in Antigenicity of the Biotin-TPO Label



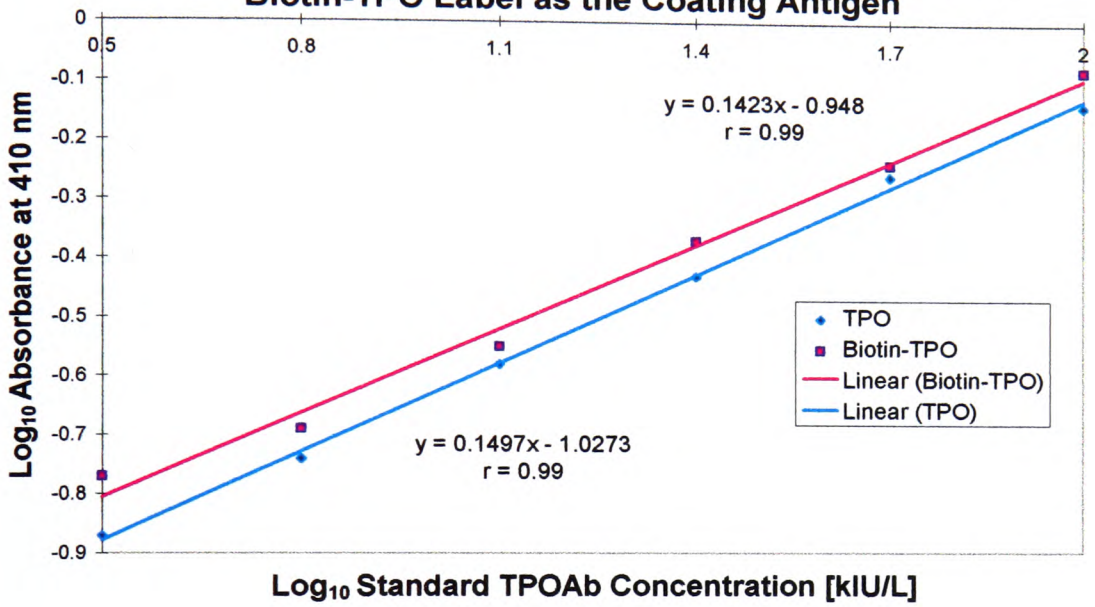
Experiment 2

An alternative experiment to investigate any change or loss in the antigenicity in the TPO after biotinylation was also set up by coating the upper section (rows A-D) of an ELISA micro-titre plate with 1 µg/ml crude TPO per well as is used in the ELISA (Groves *et al.*, 1990). The crude TPO (approximately 10 mg/ml) was diluted accordingly with coating buffer for use. The lower section (rows E-H) of the micro plate was coated with the Biotin-TPO label, similarly diluted with coating buffer. The ELISA was then carried out [Appendix 2:7], and the response of the labelled and unlabelled TPO preparations compared.

The logarithmically converted data was plotted and results compared [see Fig 36]. The Biotin-TPO label produced a response comparable with that of the unlabelled TPO [correlation coefficient (r) = 0.99], which again indicated no apparent loss of antigenicity. However, the Biotin-TPO label produced

higher blank values and also falsely elevated values (almost double) as compared with the unlabelled TPO for a 'Normal' control. The anomalous reponse was confirmed by repeat analysis for this particular 'Normal' quality control sample, but was not evident in an alternative 'Normal' control.

Figure 36
Graph of ELISA Comparing TPO (Unlabelled) and the Biotin-TPO Label as the Coating Antigen

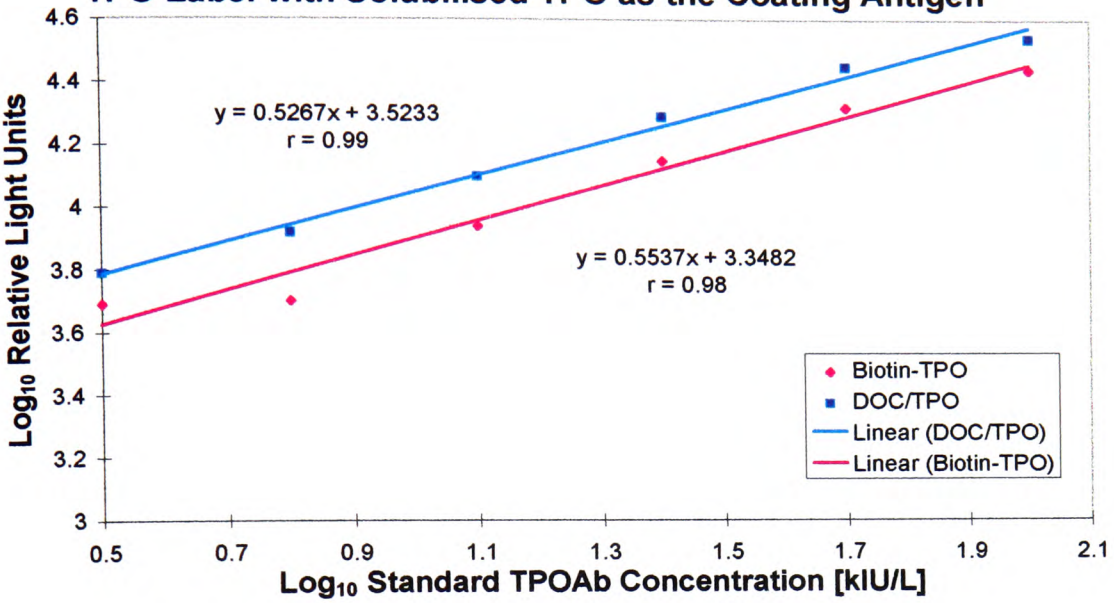


Experiment 3

The Biotin-TPO label was also used as the coating antigen replacing the solubilised TPO in the coated tube assay [see 2.8.3]. The Biotin-TPO and solubilised TPO were diluted as described in Experiment 2 above. The coated-tube assay was carried out using the sheep anti-human IgG acridinium label (diluted 1:1000) and a good curve response was produced [correlation coefficient (r) of 0.98 (on two separate assays)], indicating the viability of the Biotin-TPO label in this assay also [see Fig 37].

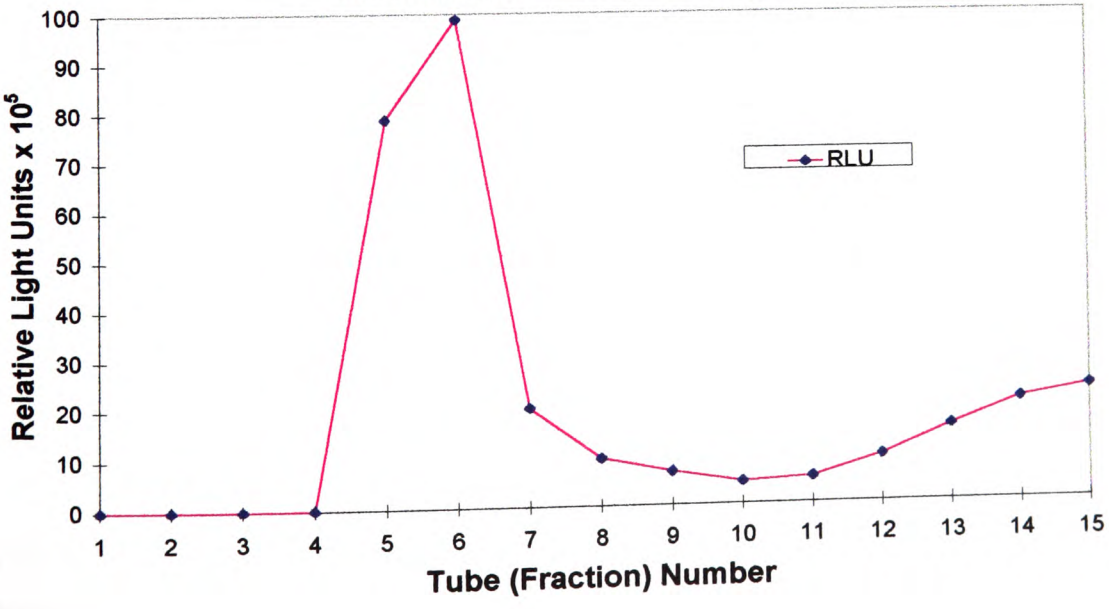
A similarly anomalous result was produced for the 'Normal' control in this experiment, again indicating that an acquired cross-reactivity of the TPO could have occurred upon biotinylation.

Figure 37
Graph of Coated-Tube Assay: Comparing the Biotin-TPO Label with Solubilised TPO as the Coating Antigen



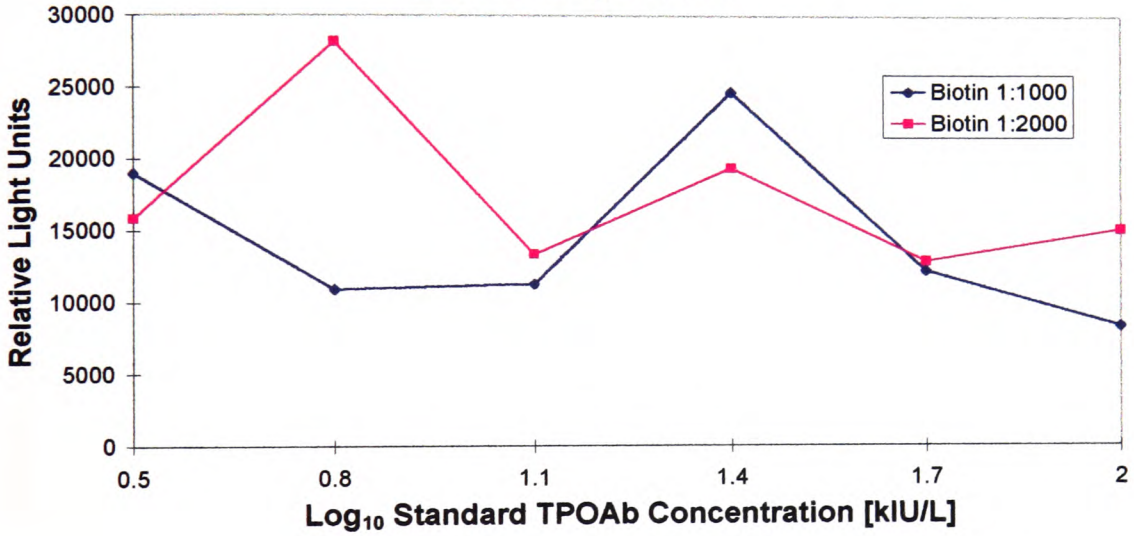
The IgG was labelled with AE [see 2.7.3 and purified via gel filtration using Sephadex™ G-50M and eluted in fractions 5 and 6, which was consistent with previous TPO labelling procedures [see Fig 38 for the elution profile].

Figure 38
Graph Showing the Elution Profile of IgG Labelled with Acridinium Ester (Acrid-IgG)



The results indicated the assay system to be non-functional with a non-uniformity of reponse of the standards which exhibited substantial oscillation with the Biotin-TPO labels (1:1000 and 1:2000) and the Acrid-IgG label initially diluted 1:2000 [see Fig 39]. A series of 'L4' control dilutions also assayed using the Biotin-TPO label (diluted 1:100 and 1:1000) and the Acrid-IgG label (diluted both 1:1000 and 1:2000) exhibited a 'blanket' response with very little difference in the RLUs between the different dilutions (data not shown).

Figure 39
Graph of Assay Method 3 (System 2): Using Biotin-TPO and Acrid-IgG Label



3.4.2 Experiments to Investigate the Viability of Assay Components

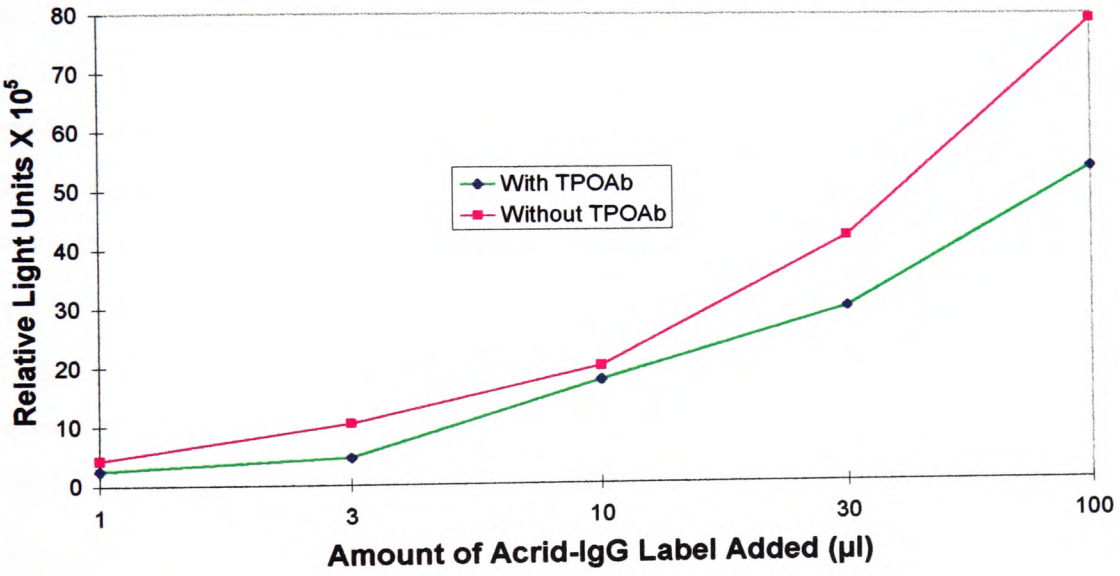
Experiment 1: Investigation of the Competitive Reaction between the Acrid-IgG Label and Sample MicAb/TPOAb

This experiment involved incubating 100 µl of the Biotin-TPO label (diluted 1:100 with TBS, containing 1% sheep serum) [Appendix 1:4] with increasing amounts of Acrid-IgG label, (i.e. 1, 3, 10, 30, 100 µl) (diluted in a a high TPOAb titre sample, prediluted 1:100) being added to each tube in a total volume of 100 µl. Similarly, another set of tubes was set-up with increasing amounts of Acrid-IgG label, but the label was diluted in diluent excluding the

high TPOAb sample. The tubes were incubated at room temperature for 2 hours, and then 20 µl (in excess) of the washed streptavidin beads were added and the tubes were incubated for a further hour at room temperature. The separated beads were washed and decanted 4 times with ELISA wash solution [Appendix 1:5], and the RLUs attached to the beads were measured on the Bayer/Chiron Magic Lite Luminometer.

The two sets of tubes exhibited a similar response, indicating that the addition of the high TPOAb sample had no or little effect/interference on the interaction of the components within the experimental system [see Fig 40].

Figure 40
Graph Showing the Effect of the Addition of a High TPOAb Sample to the Acrid-IgG Label



Experiment 2: Investigation of the Uptake of the Biotin-TPO Label by the Streptavidin-labelled Beads

The Biotin-TPO label was diluted 1:100 with TBS/1% sheep serum, and 250 µl of the diluted label was placed into a plastic tube. An excess of 80 µl of washed streptavidin-labelled beads was added and the contents vortex mixed and incubated at room temperature for 1 hour. The resulting supernatant was diluted (in a serum sample containing a high TPOAb concentration), so that increasing concentrations of the supernatant were

added across an ELISA plate. The experiment was carried out on two separate Biotin-TPO label fractions, and a PBS blank and 'normal' and 'high' QC were included to ensure optimal ELISA assay conditions.

There was no decrease in absorption at 410nm with increasing addition of supernatant and this indicated that the streptavidin beads had successfully removed the Biotin-TPO from the supernatants (data not shown).

Experiment 3: Experiment to Further Investigate the Functional Capacity of the Streptavidin-labelled Beads

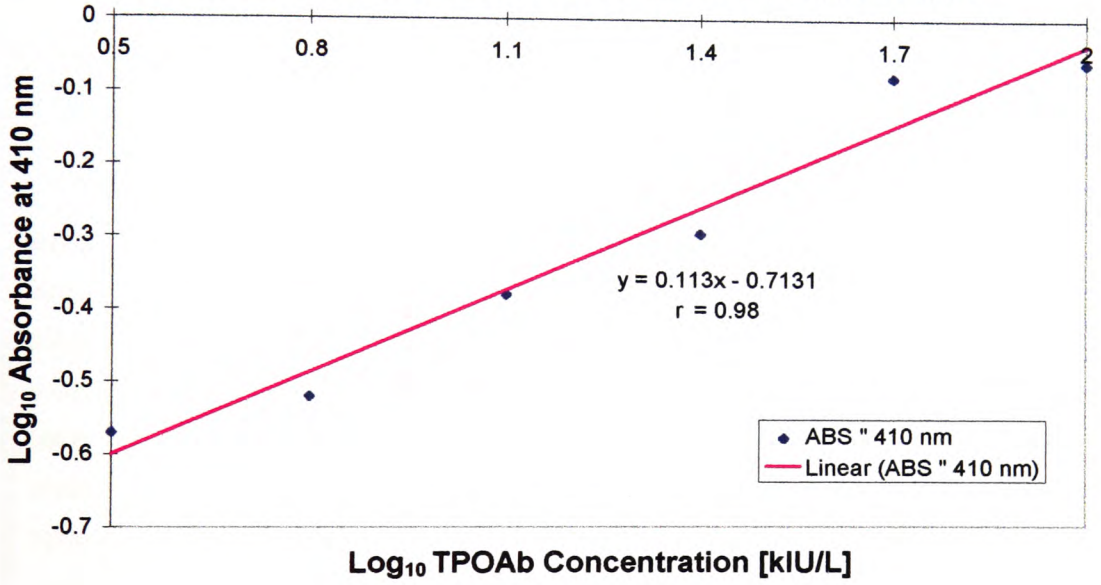
Appropriately diluted standards, quality controls plus a PBS blank [see 2.7.5], were pipetted in 100 µl aliquots (in duplicate) into plastic Sarstedt tubes. To each tube, was added 100 µl each of the Biotin-TPO and Acrid-IgG label (both diluted 1:100 in label diluent). The tubes were incubated for 2 hours at room temperature and then 20 µl of washed streptavidin beads were added and further incubated for 1 hour. The beads were decanted and washed four times with ELISA wash [Appendix 1:5].

Then, instead of measuring the RLUs of the acridinium ester (attached to the beads), the final ELISA quantitation stage for the measurement of TPOAb was applied, using the colorimetric antibody/substrate reaction as described next.

To each tube containing the washed magnetic particles, 100 µl of freshly prepared conjugate solution (i.e. peroxidase conjugated sheep anti-human IgG antibody), (sheep antiserum obtained from Serotec, Oxford, U.K.) was added [Appendix 1:5]. The tubes were vortex mixed and then incubated at room temperature for 30 minutes at room temperature. The particles were washed 4 times with ELISA wash [Appendix 1:5], and then 100 µl of the ELISA substrate solution ABTS [2,2' Azino-bis(3-ethylbenzthiozoline-6-sulphonic acid)] (Boehringer Mannheim) was added. The tubes were then incubated at room temperature with the top standard being carefully monitored and the reaction stopped by adding 50 µl of stopping reagent [2% sodium fluoride (NaF)], when the colour intensity appeared to be comparable

with that of the ELISA. The green supernatant (140 µl) was then removed from each of the tubes and pipetted into an ELISA microtitre plate and the absorbances read at 410 nm on the Dynatech (MR5000) Multi-plate reader. [The ELISA method used was as described by Groves *et al.*, 1990, which in turn was based on the method of Voller *et al.* (1980), see Appendix 2:7]. The logarithmically converted absorbances measured at 492 nm produced a good regression curve response [correlation coefficient (r) of 0.98] [see Fig 41], and the 'Normal' control produced an erroneously high result as demonstrated before [see 3.4.1: Experiments 1 & 2].

Figure 41
Graph of Method 3 (System 2) with the Final Quantitation Using the ELISA Colorimetric Procedure



The results obtained, implicated the source of the problem as being the lack of purity of the IgG (purified as described in Appendix 2:6] used in the preparation of the Acrid-IgG label [see 2.7.3, 2.6.2 & 2.6.4]. The label did not appear to be competing effectively in the assay system, so it was decided to further purify the Acrid-IgG label using CNBr-activated Sepharose™ 4B [see 2.7.6]. The non-covalently bound components were eluted in TBS azide [see Fig 42] and the final elution of the label achieved using a 3M sodium thiocyanate solution [see Fig 43] (an initial attempt to elute the purified label with 2M NaCl was unsuccessful). The label was identified by measuring the RLUs of each of the diluted 1 ml fractions.

Figure 42
Graph Showing the Elution of Non-Specifically-Bound Components in the Purification of Acrid-IgG Label (Using TBS Azide)

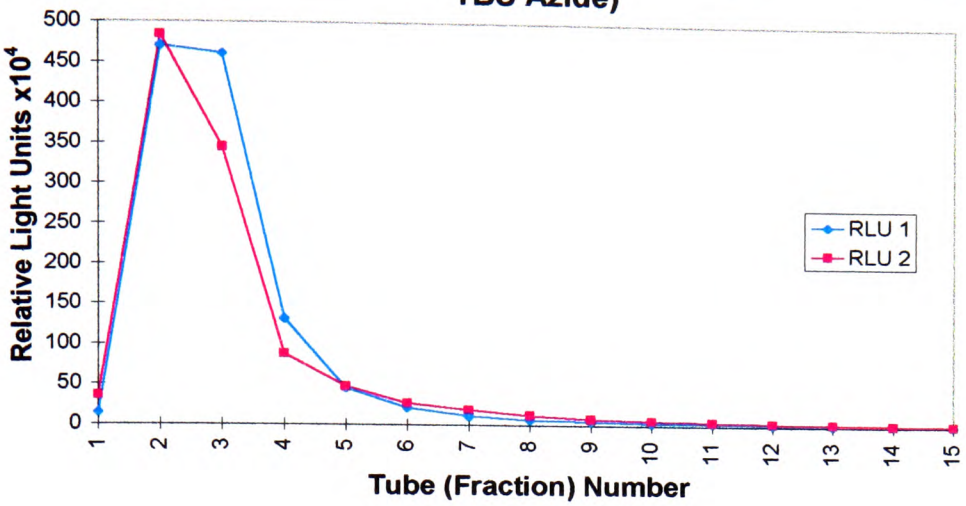
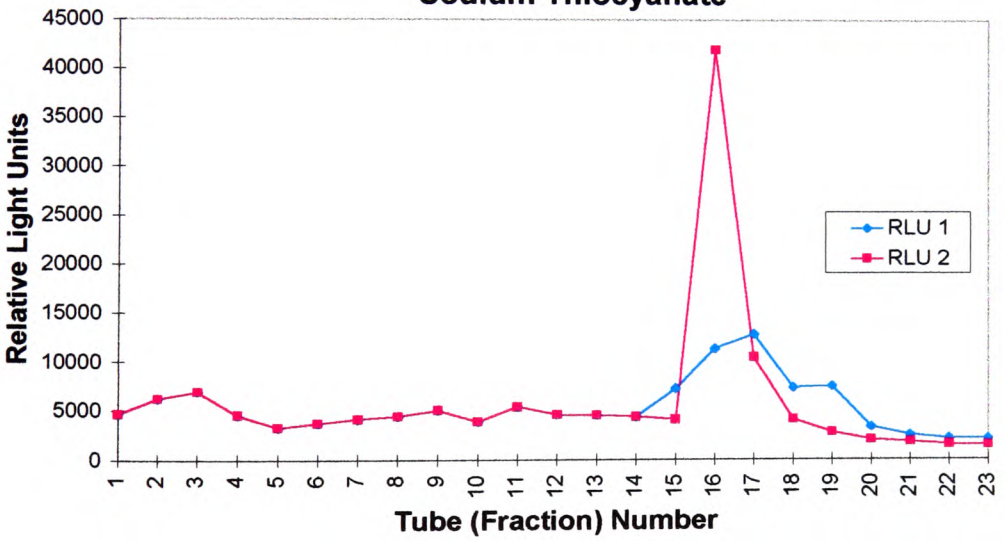


Figure 43
Graph Showing the Elution of Purified Acrid-IgG Label in 3M Sodium Thiocyanate

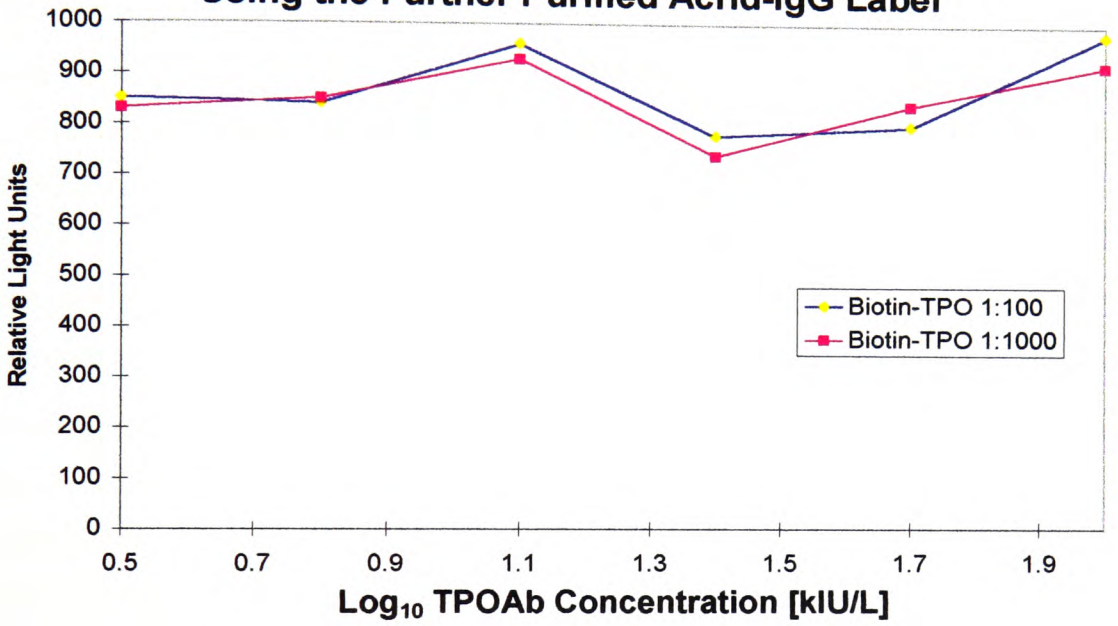


Figures 42 & 43 The elution profile of the further purified Acrid-IgG label.

The label was not eluted in 2M NaCl (Fractions 1 to 14), but in 3M sodium thiocyanate (Fractions 16 and 17) [see Fig 43].

Two attempts were made to further purify the Acrid-IgG label, with an apparently improved yield on the second attempt [see Fig 43]. However, assays using this purified label [see 2.7.7], served to provide no improvement in the assay, with no apparent uptake of AE by the magnetic beads at all, resulting in little difference (in RLUs) with consecutive standards [see Fig 44].

Figure 44
Graph of Standard Curve for Method 3 (System 2) Using the Further Purified Acrid-IgG Label



3.5 Method 3 (System 3)

Solid-Phase Chemiluminometric Assay Using Sheep Anti-Human IgG Labelled With Acridinium Ester (Coated-Tube Assay)

This assay procedure [see 2.8.3] with the immobilisation of the solubilised TPO antigen onto plastic tubes, provided a direct quantitation of the sample TPOAb in RLUs, using the sheep anti-human IgG acridinium label (Acrid-Anti-IgG). The Acrid-Anti-IgG label was prepared via the same procedure as used for the Acrid-IgG label [see 2.7.3 & 2.8.2] and was eluted in fraction 6 (data not shown).

This assay demonstrated superior precision (analyses in duplicate) as compared with the other chemiluminescent assay systems. Assays were carried out using the Acrid-Anti-IgG label initially diluted 1:1000 [see Fig 45 for the standard curve of Relative Light Units versus Log_{10} TPOAb concentration (error bars being defined as 1 S.D.) and Fig 46 for the logarithmic plot].

Assays using the Acrid-Anti-IgG label diluted 1:2000 [correlation coefficient (r) of 0.98], indicated a slight compromise in assay performance with an uptake at best of 18,880 RLUs (i.e. log_{10} value = 3.5) by the top standard [see Fig 47], as compared with an uptake of 53,900 RLUs (i.e. log_{10} value = 4.73) by the top standard with the 1:1000 dilution ratio [coefficient correlation (r) of 0.99].

Assays with the final incubation reduced from 1 hour to 30 minutes (Acrid-Anti-IgG label diluted 1:1000) produced comparable correlation coefficient (r) value of 0.99 upon regression curve analysis, but again a reduced uptake of 24,570 RLUs (i.e. log_{10} value = 3.65) was demonstrated by the top standard [see Fig 48]. Therefore, in future studies a 1:1000 dilution of the Acrid-Anti-IgG label was used with an incubation period of 1 hour.

Figure 45
Graph of Coated-Tube Assay Using Sheep Anti-human IgG Labelled with Acridinium Ester [Method 3 (System 3)]

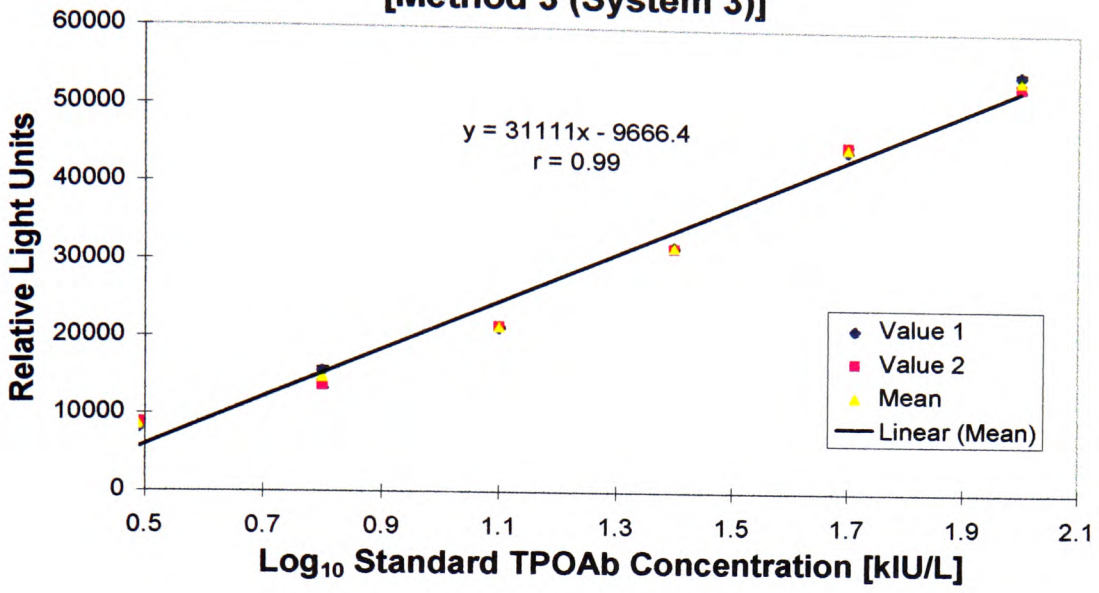


Figure 46
Logarithmic Graph of Coated-Tube Assay [Method 3 (System 3)]

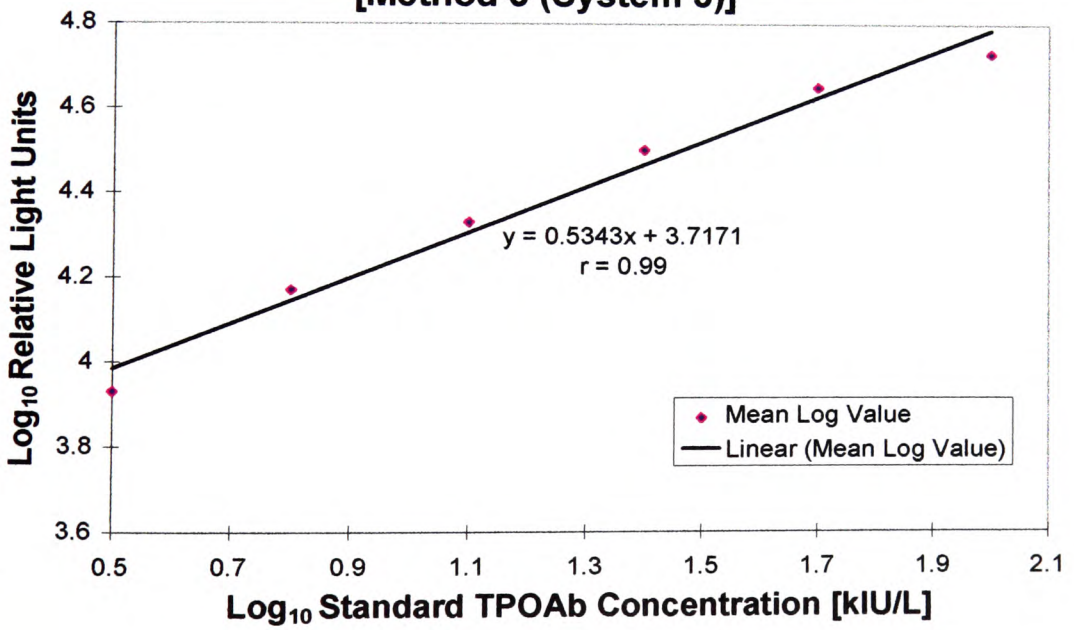


Figure 47
Graph of Coated-Tube Assay Using the Sheep Anti-human IgG-Acridinium Label (Diluted 1:2000)

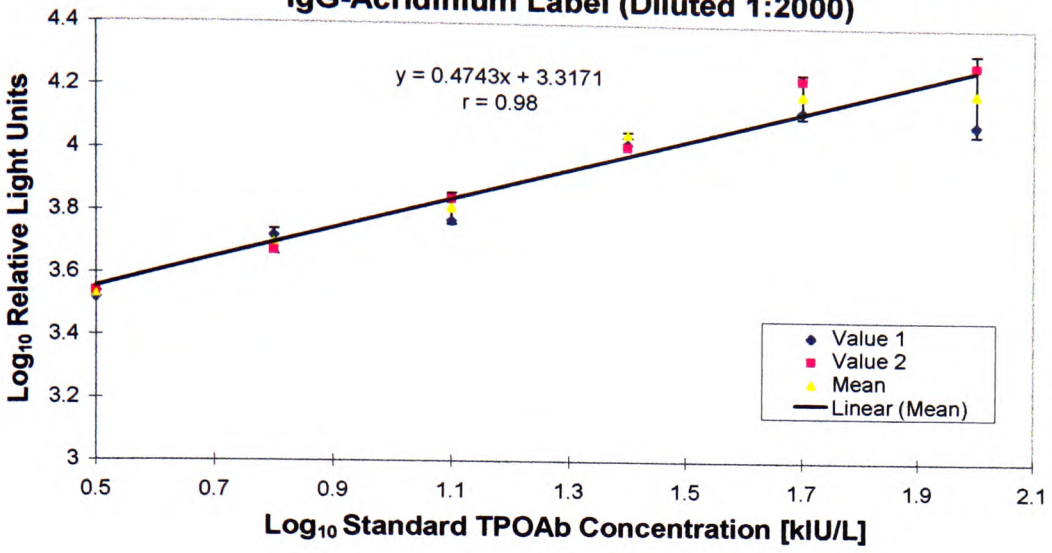
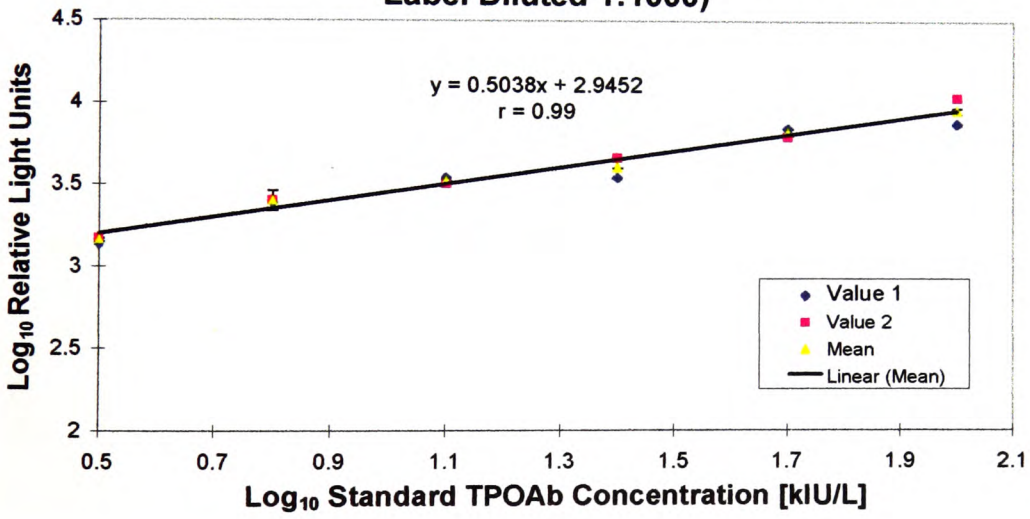


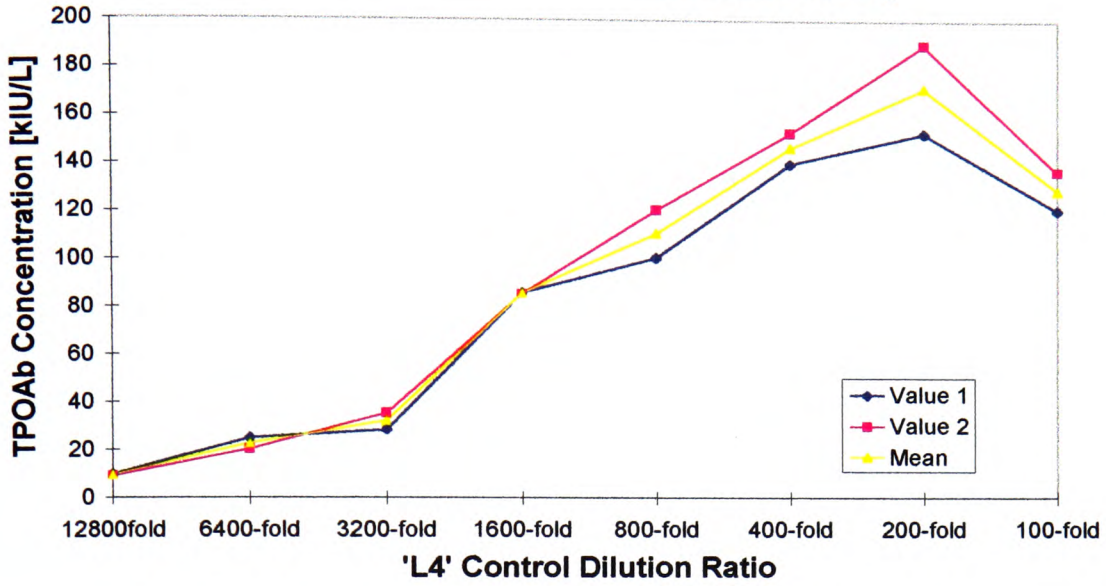
Figure 48
Graph of Coated-Tube Assay with a Final Incubation Period of 30 Minutes (Sheep Anti-human IgG-Acridinium Label Diluted 1:1000)



3.5.1 Experiment to Determine the 'High-Dose Hook' Effect

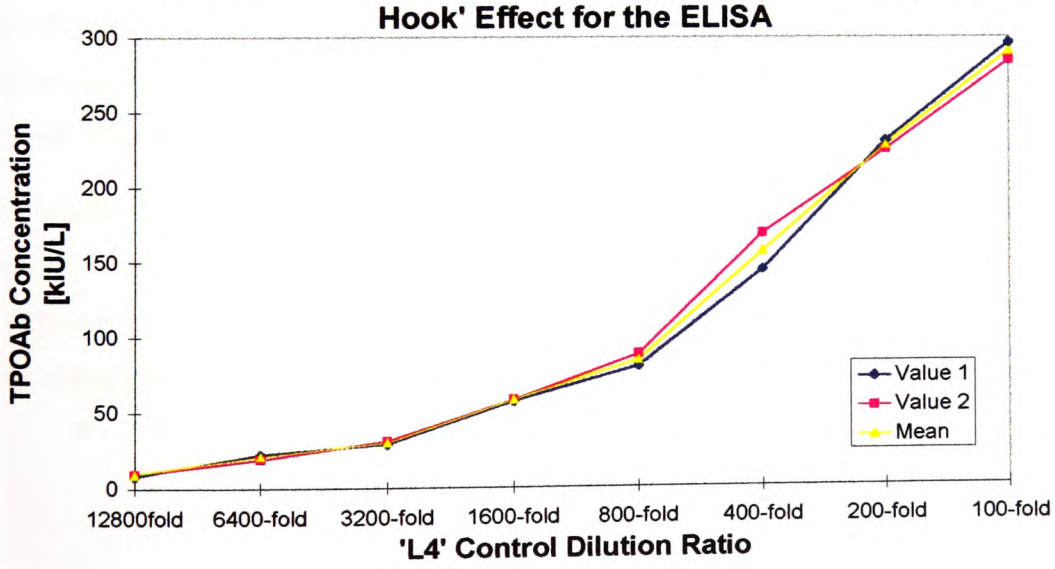
The 'high-dose hook' effect was investigated by assaying different dilutions of the high 'L4' control (analyses in duplicate) and was found to manifest at a 200-fold dilution (i.e. an approximate mean TPOAb value of 172 kIU/L) for the coated-tube assay, but increased imprecision was evident at the higher TPOAb concentrations which slighlty obscured results [see Fig 49].

Figure 49
Graph of 'L4' Control Dilutions to Investigate the 'High-dose Hook' Effect for the Coated-Tube Assay



A series of 'L4' control dilutions were also assayed via the ELISA [see Fig 50].

Figure 50
Graph of 'L4' Control Dilutions to Investigate the 'High-Dose Hook' Effect for the ELISA



The threshold for the 'high-dose hook' effect in the ELISA was demonstrated to be higher than in the coated-tube assay, with no effect exhibited at an 'L4' dilution ratio of 100-fold, as compared with 200-fold for the coated-tube

assay. The ELISA demonstrated better precision at higher TPOAb concentrations.

3.5.2 Determination of the Analytical Sensitivity

The 'analytical sensitivity' was derived using the criteria used by Bayer/Chiron Diagnostics [see 2.8.4]. An analytical sensitivity of 1.8 kIU/L was calculated using 20 replicates of the PBS diluent 'blank' assayed within the same run.

However, 'blank' values from 13 consecutive assays ('between-run' data) gave an analytical sensitivity of 2.46 kIU/L. This value would encompass the 'between-run' or 'batch to batch' variation demonstrated in the 'blank' measurements.

3.5.3 Estimation of the 'Within-Run' (Intra-Run) Precision and 'Between-Run' (Inter-Run) Precision

The 'within-run' precision was determined by assaying 20 replicates each of a 'Normal' control and a 'High' control in the same coated-tube assay and the standard deviation (S.D.) and the coefficient of variation (C.V.) for each control was calculated [see Table 3].

Similarly, the S.D. and C.V. was calculated for the 'Normal', 'High' and 'L4' quality control data produced from 13 consecutive assays, to determine the 'between-run' precision [see Table 3]. Precision studies were also performed for the ELISA [see Table 4].

Table 3 Precision Data for the Coated-Tube Assay

	'Within-Run' (Intra-Run) Precision		'Between-Run' (Inter-Run) Precision		
	'Normal Control'	'High' Control	'Normal' Control	'High' Control	'L4' Control
Number	20	20	13	13	13
Mean kIU/L	4.62	77.8	5.10	68.0	179
S.D.	1.32	8.48	1.91	8.90	22.9
C.V. (%)	28.6	10.9	37.5	13.1	12.8

Table 4 Precision Data for the ELISA

	'Within-Run' (Intra-Run) Precision		'Between-Run' (Inter-Run) Precision	
	'Normal Control'	'L4' Control	'Normal' Control	'L4' Control
Number	4	4	10	10
Mean kIU/L	11.70	373.0	4.40	225.0
S.D.	1.61	28.50	1.55	26.48
C.V. (%)	13.8	7.7	35.2	11.8

3.5.4 Assessment of Assay Interferences (Assay Specificity)

Various antibodies were measured via the coated-tube system to see if they caused any significant interference. They included, anti-nuclear factor (ANF), smooth-muscle antibody (SMA), and varying titres of rheumatoid factor (RF).

Samples with different degrees of positivity of rheumatoid factor did not appear to cause any significant interference with the coated-tube assay or the ELISA. Anti-nuclear factor (antibody) (ANF/ANA) and smooth-muscle antibody (SMA) also did not cause any significant interference in the coated-tube assay. SMA interference was evident in the ELISA, but its interference could be attributed to the highly icteric nature of the sample, which interfered with the ELISA colorimetric measurement.

Grossly haemolysed, lipaemic, and icteric (jaundiced) samples were investigated for any significant interference in the coated-tube assay [see Table 4]. The samples were assayed (in duplicate) both neat and diluted by 50% with a TPOAb negative sample (with the latter TPOAb sample demonstrating a mean RLU value of 2000 on duplicate analyses). The samples were diluted 100-fold with PBS, pH 7.4, as for patient samples and assayed. No significant effect was demonstrated in the coated-tube assay, indicating no (positive) interference of any of the samples studied. However, the icteric sample (which was also MicAb/TPOAb negative via indirect agglutination), did cause positive interference in the ELISA, giving a TPOAb titre of 39 kIU/L.

Table 5 Results of Investigation of Assay Interferences in the Coated-Tube Assay

	Neat Sample		Sample Diluted 50% with TPOAb Neg Sample	
Sample	**RLU**	**kIU/L**	**RLU**	**kIU/L**
Haemolysed	1430	1.1	1780	1.8
	1790	1.8	1760	1.7
Lipaemic	1980	2.3	2440	3.6
	1930	2.1	2330	3.3
Icteric	2440	3.6	1770	1.7
	2330	3.3	1890	2.0

3.5.5 Comparison Studies

(i) Coated-Tube Assay versus ELISA

The data obtained from 100 patient sera were compared for the coated-tube assay and the ELISA. The samples were obtained from a biased population, which inferred that the population would be of non-Gaussian distribution (i.e. a Gaussian distribution is when 95% of the values are between +/- 1.96 S.D.).

This was confirmed by the analysis of the data using Minitab: Version 9.

Coated-Tube Assay: Std. Dev = 78.89, Mean = 86.8, n = 100

ELISA: Std. Dev = 76.33, Mean = 89.7, n = 100

The data were analysed using three statistical models and the logarithmically-transformed data indicated a very good correlation between the coated-tube assay and ELISA for all statistical models used. A correlation coefficient (r) of 0.96 and (P= <0.001) was produced using the 'least squares' regression method [see Fig 51]. The data was also evaluated using the Deming Method and the Passing & Bablok Method. Similar slope values of 1.04 and 1.01 (with ranges of 0.99 to 1.08 and 0.96 to 1.05) were achieved respectively for the regression analyses [see Figs 52 & 53].

Figure 51
Comparison of Coated-Tube Assay and ELISA Using the 'Least-Squares' Regression Method

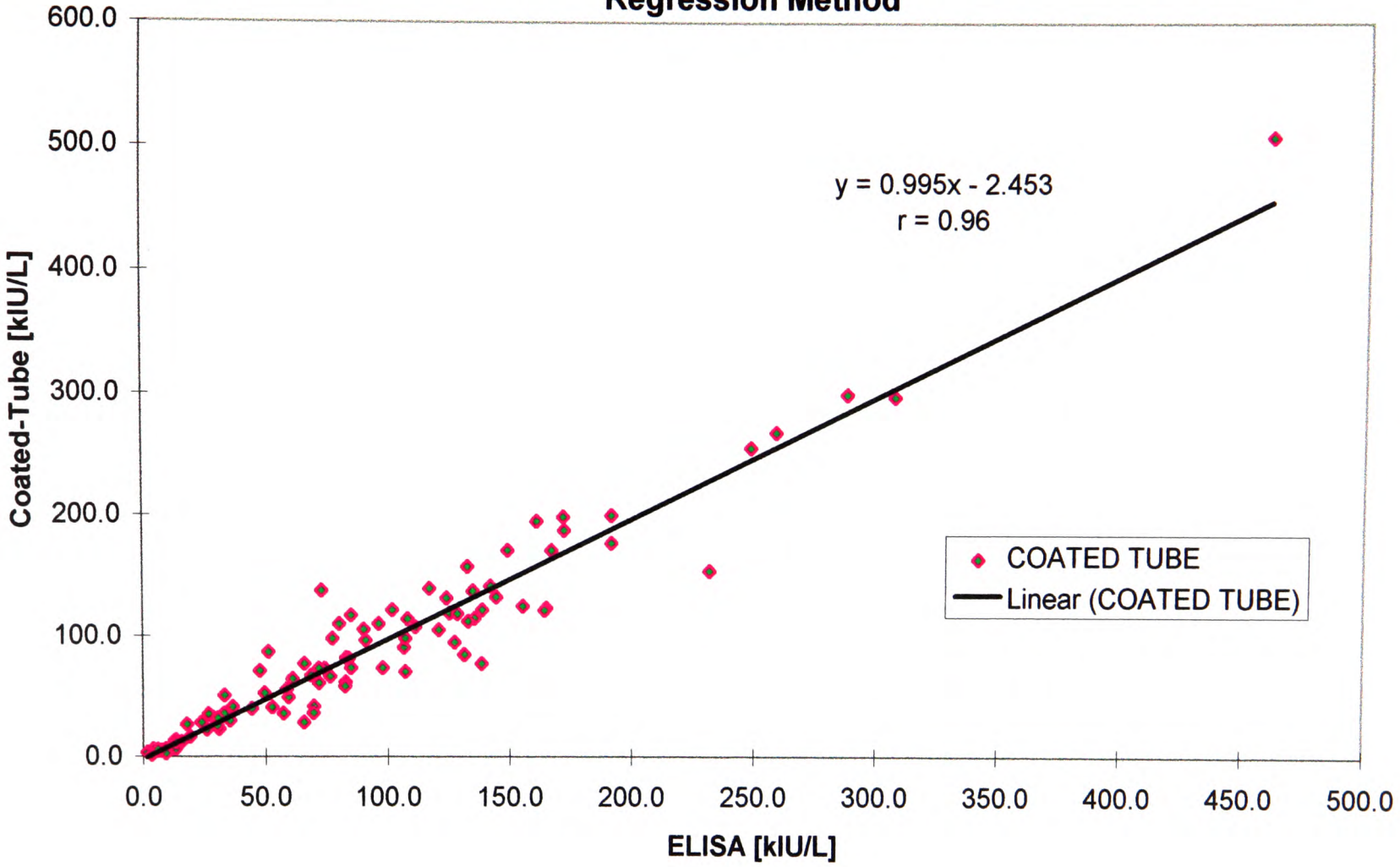


Figure 52 (i)

Deming Method Comparison of Coated-Tube Assay with ELISA

n	100	
Imprecision SD	1.0000	1.0000
Variance ratio	1.0000	

	Coefficient	SE	90% CI	
Intercept	-6.0423	3.4596	-11.7872 to -0.2974	(constant bias detected)
Slope	1.0350	0.0294	0.9861 to 1.0839	

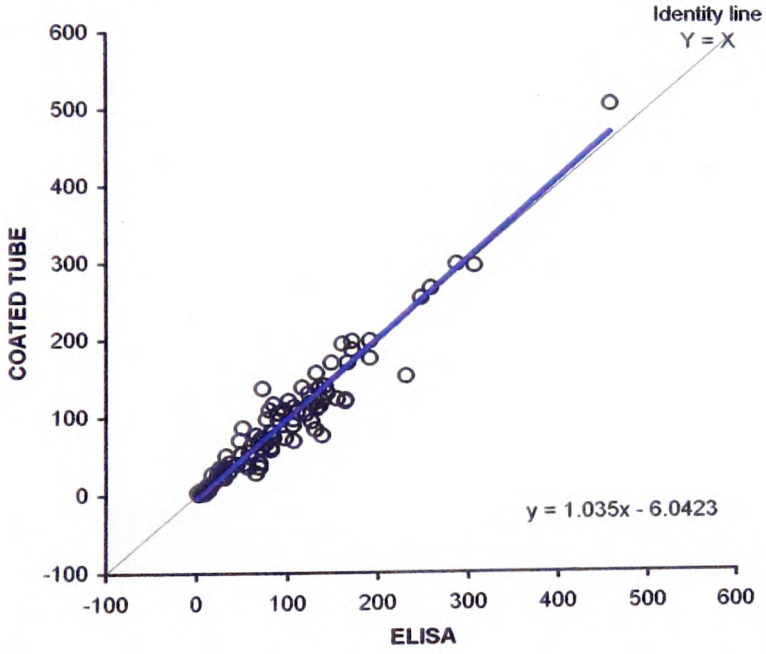


Figure 52 (ii)

Deming Method Comparison of Coated-Tube Assay with ELISA - Graphic Representation of Residual Values

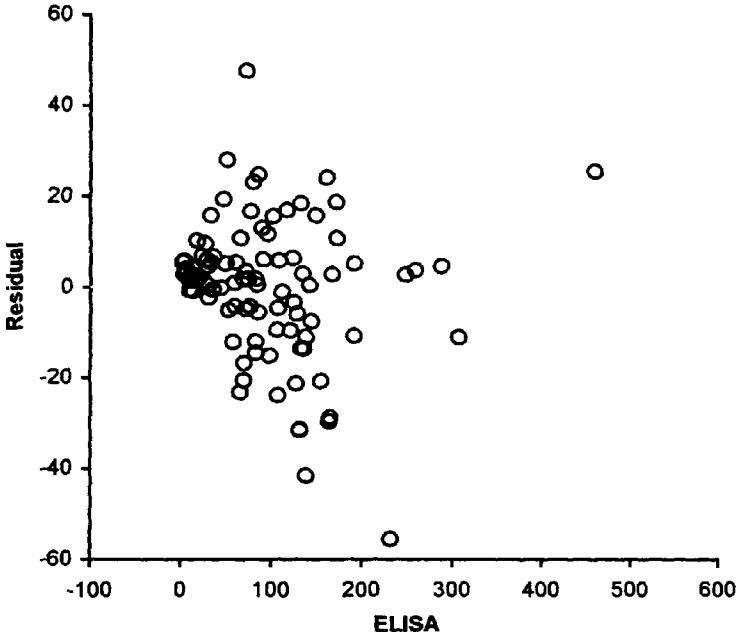


Figure 53 (i)

Passing & Bablok Method Comparison of Coated-Tube Assay and ELISA

n	100

	Coefficient	90% CI
Intercept	-2.001	-4.191 to 0.109
Slope	1.015	0.955 to 1.053

Cusum test for linearity - p	> 0.1

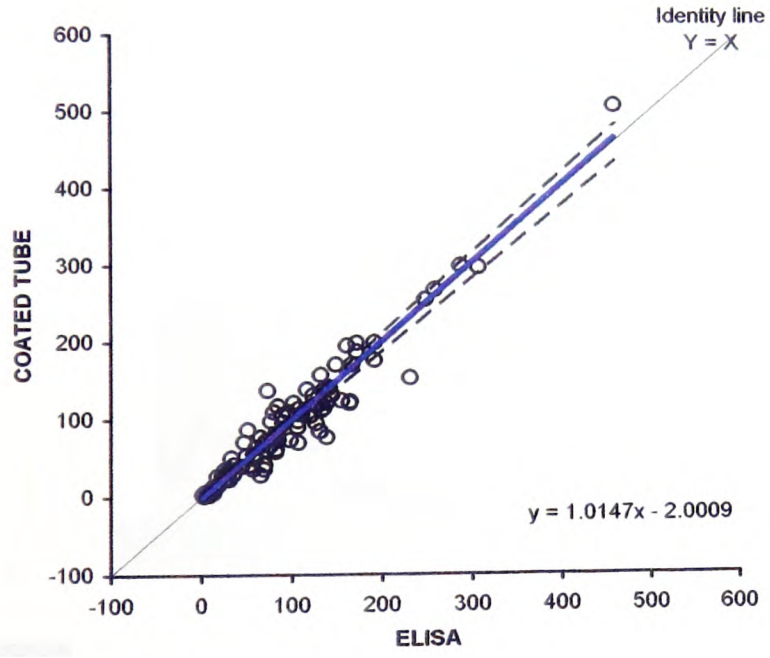
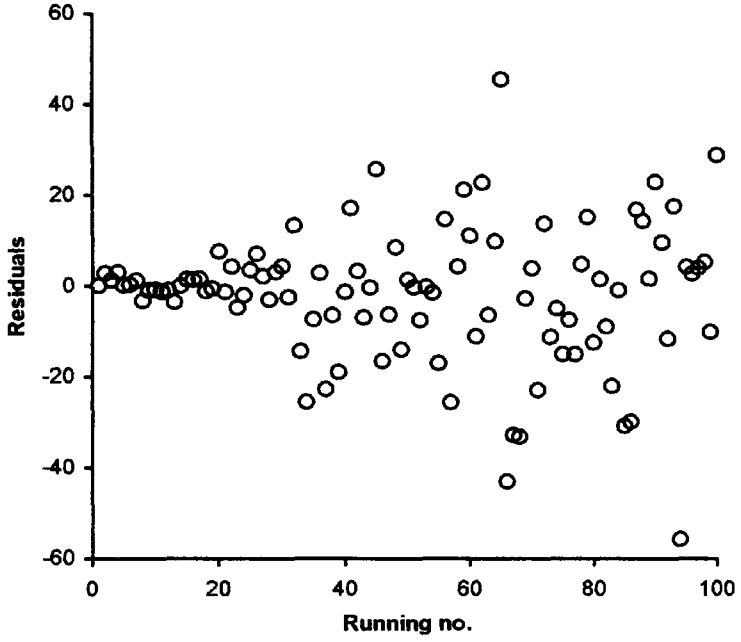
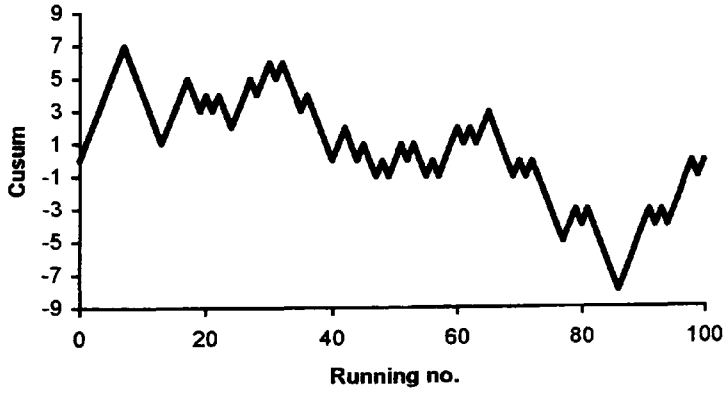


Figure 53 (ii)

Passing & Bablok Comparison of Coated-Tube Assay and ELISA - Graphic Representation of Residual Values





Clinical Specificity / Sensitivity of the Coated-Tube Assay

One discordant result was produced which gave a borderline positive result for the chemiluminescent coated-tube assay as compared with a borderline negative result in the ELISA (assuming a 'cut-off' value of 19.6 kIU/L, as used in the ELISA for both assays) [Table 6].

Table 6

100 samples were compared with ELISA

	ELISA Positive	ELISA Negative
Chemiluminescence Positive	80	1
Chemiluminescence Negative	0	19

Sensitivity of CIA = 100%; Specificity of CIA = 95 %

(ii) Comparison of the ELISA and Coated-Tube Assay with Indirect Agglutination

Only 37 samples were compared in this study with the agglutination assay values being rank ordered as follows for graphical representation:

1 - Negative; 2 - 1:100; 3 - 1:400; 4 - 1:1,600; 5 - 1:6,400; 6 - 1:25,000; 7 - 1:100,000 and 8 - 1:500,000.

The logarithmically converted data for both the coated-tube assay and the ELISA compared well with the indirect agglutination assay, with correlation coefficients (r) values of 0.80 and 0.85 respectively [see Figs 54 & 55]. The application of the 'Wilcoxon-Mann-Whitney U Test' indicated that the correlation of data for both the coated-tube assay and ELISA with indirect agglutination was significant (P = <0.0001). However, the graphs indicated a tendency for the indirect agglutination assays to underestimate at high concentrations and be less sensitive at lower concentrations (with three samples giving negative titres for the indirect agglutination assay, but

positive titres for both the coated-tube assay and the ELISA) (with a titre of 1:100 considered negative in the indirect agglutination assay, and a 'cut-off' value of 19.6 kIU/L being applied in the other assays) [Table 7].

Table 7

37 samples were compared with Indirect Agglutination

	Agglutination Positive	**Agglutination Negative**
Chemiluminescence Positive	26	3
Chemiluminescence Negative	0	8

As CIA was identical to ELISA for these samples both agglutination and CIA were 100% specific. CIA was also 100% sensitive but agglutination only 73% sensitive.

Figure 54
Comparison of Coated-Tube Assay and Indirect Agglutination

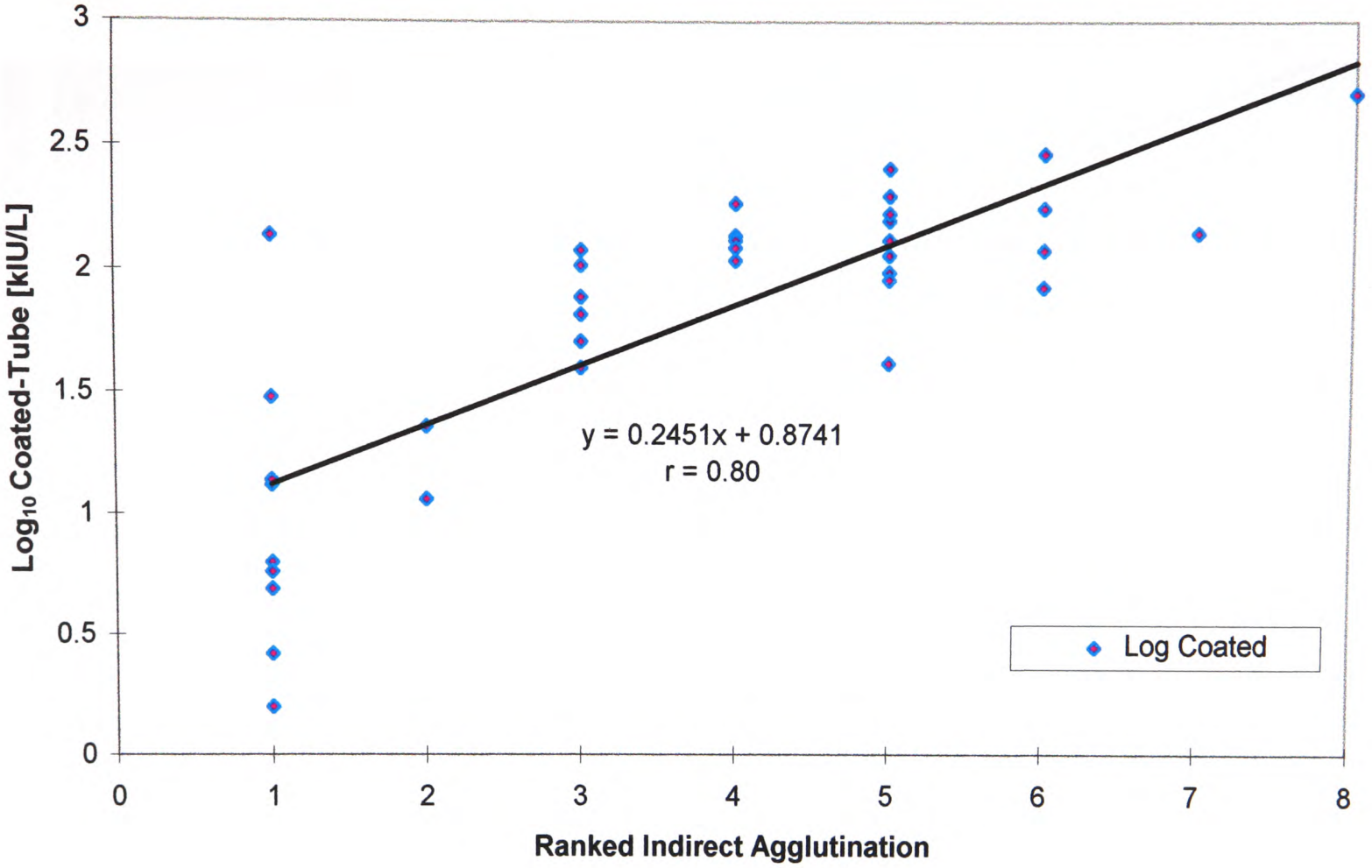
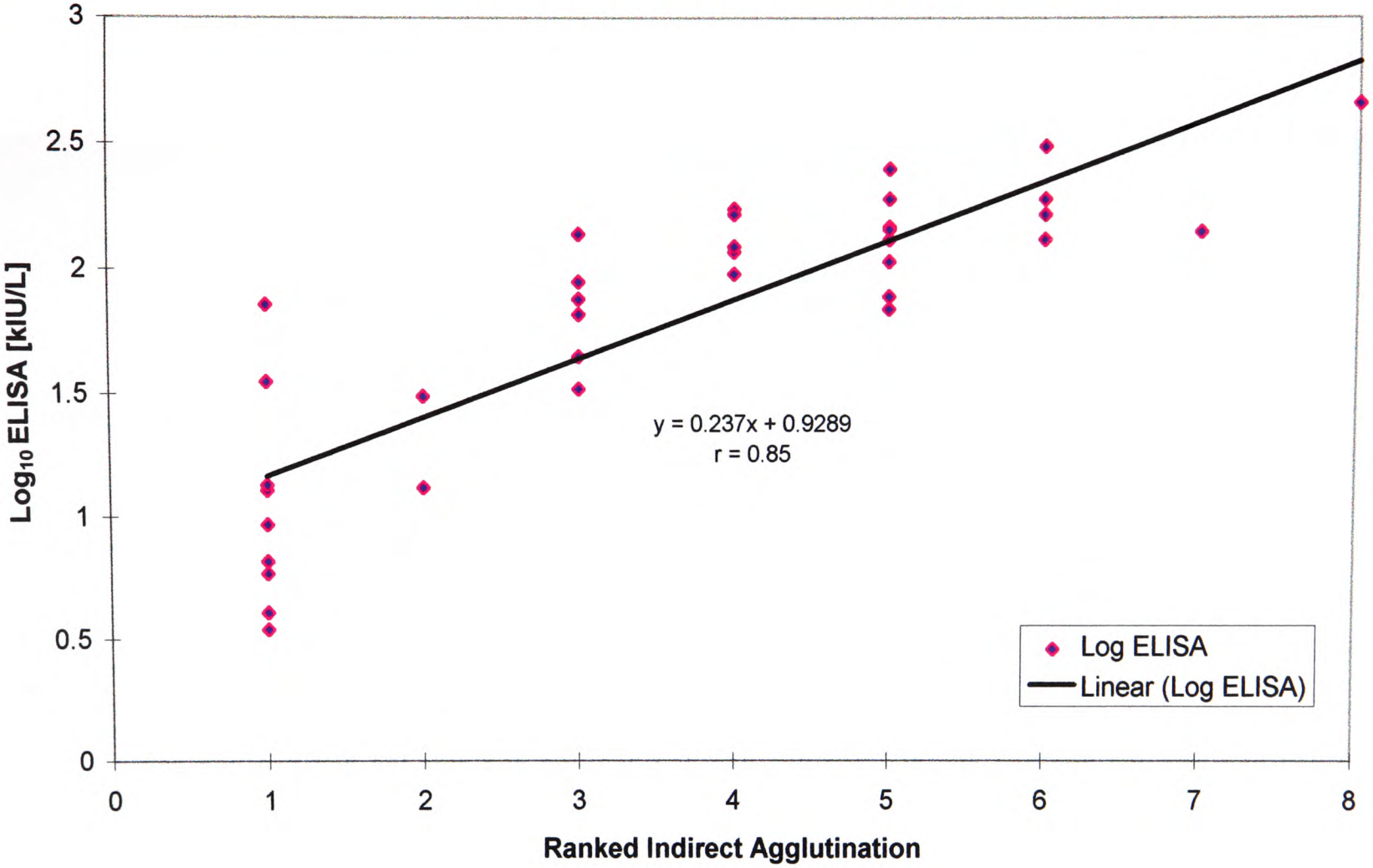


Figure 55
Comparison of ELISA and Indirect Agglutination



CHAPTER 4

DISCUSSION

Chapter 4: Discussion

The discussion will commence with a summary of the results of the five assays investigated, followed by a further detailed discussion of the various assay components and their viability within the assays.

The Immunoradiometric Assay (IRMA) [2.4: Method 1]

This assay involved the use of a commercial preparation of hTPO labelled with [^{125}I], and a Protein A magnetic suspension to effect the separation of the radiolabelled TPO-TPOAb complex. The assay system was based on the direct interaction between the [^{125}I]-labelled TPO autoantigen and autoantibody (TPOAb) (similar to an IRMA described by Beever *et al.*, 1989) and succeeded in overcoming the limitations of indirect measurement as used in agglutination, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). A full evaluation of the IRMA was not performed, as the primary aim was to demonstrate the efficacy of the Protein A suspension, which could subsequently be applied to a non-radioactive enzyme-immunometric assay system [2.5: Method 2].

The magnetic particles were successfully coupled with Protein A, with an average uptake of 94% being achieved on two preparations. No advantage was gained for the further uptake of IgG by the suspension with incubation overnight at 37°C as compared with incubation at room temperature [Figs 15 & 16]. The Protein A suspension exhibited a high uptake of the total radioactive counts for the top standard in the IRMA (60%), a favourable assay characteristic, which augured well for a reliable, clinical assay. Results indicated that hourly incubation periods at room temperature were optimal conditions for both the initial interaction (between TPO and TPOAb), and for the final incubation period with the Protein A suspension [Fig 20]. The 'high-dose hook' effect was demonstrated with the Protein A suspension dilution ratio of 1:10, which produced the paradoxically low (and misleading) results for high TPOAb concentrations [Fig 20]. An optimum Protein A suspension assay dilution ratio of 1:5 was indicated (with a 200-fold sample dilution) [Fig 21].

The limited results compared favourably with indirect agglutination [Table 1: 3.1] endorsing the findings of Beever *et al.* (1989). The TBS blank consistently demonstrated values significantly lower than the lowest TPOAb standard (of 1 U/L), which indicated a low degree of non-specific binding and hence a high assay sensitivity. This was exemplified in Sample 5, with the IRMA identifying low TPOAb titres in contrast to negative titres by indirect agglutination. A discordant negative result was also reported in the IRMA for Sample 1, as opposed to a positive titre of 1:400 for indirect agglutination [Table 1: 3.1]. This could indicate an increased assay specificity as compared with the indirect agglutination microsomal assay. Sample 1 was accompanied by a high thyroglobulin autoantibody (TgAb) titre of 1:12,000 and the presence of TgAb in serum has been reported as causing positive interference in certain indirect methodologies for MicAb, including passive haemagglutination (PH) microsomal assays (Cayzer *et al.*, 1978; Weetman & McGregor, 1983; Roman *et al.*, 1984; Ruf *et al.*, 1988; Mariotti *et al.*, 1990).

Although the IRMA demonstrated very good assay characteristics, due to the economic implications and restrictions of supporting the use of isotopes, radioactive methodologies in the routine laboratory environment have been widely abandoned and limited to a more specialised use, prompting the search for non-radioactive alternatives.

The Enzyme-immunometric Assay [2.5: Method 2]

This unsuccessful, non-competitive system involved the labelling of a crude (solubilised) preparation of TPO with HRP. Although, HRP activity was demonstrated in the HRP-TPO label [Fig 22], and the Protein A suspension was proved to be functional by reacting with the HRP-TPO-labelled complex (indicated by the HRP activity associated with the suspension), no discrimination between consecutive TPOAb standard dilutions was exhibited [Fig 23]. All the assay components were reprepared, but the same 'blanket' response was produced upon repeat analysis. The same response was exhibited when potassium iodide was used as the HRP substrate.

Chemiluminescent Assay [2.6: Method 3 (System 1)]

This assay involved labelling the crude (solubilised) TPO with the chemiluminescent label acridinium ester (AE), again resulting in a non-competitive assay system. The final RLUs attached to the magnetic particles (coupled to anti-human IgG) were directly proportional to the TPOAb concentration. This system was inferior to the IRMA, but did demonstrate an improvement over the enzyme-immunometric assay, and was capable of distinguishing between high and low TPOAb titres. The standard curve, however, lacked precision (especially at the higher TPOAb titres), and exhibited considerable overlapping of consecutive data points.

Varying the ratio of TPO to AE in the labelling procedure [see 2.6.3] did not improve the uptake of the top standard, with the maximum uptake of only 1.1% being exhibited with the label prepared using 0.05 µg of AE. The best standard curve parameters were achieved with the solubilised TPO label containing 5.5 µg of AE with correlation coefficients (r) of 0.99 and 0.98 being achieved on two separate assay attempts, but with the uptake of the top standard being only 0.4% and 0.5% respectively [Fig 26]. The lowest concentration of AE was accompanied by little difference in consecutive data points [Figs 27-30].

Little difference was exhibited when labels were prepared from either crude or TPO solubilised with 1% deoxycholic acid. A slight improvement was shown with the solubilised TPO label over the corresponding crude label when prepared with 5 µg of AE, but no improvement was shown at all using 0.5 µg of AE [Fig 31]. In addition, no significant difference in the results was evident using the magnetic particles diluted 1:10 instead of 1:20, with perhaps a slight improvement in precision seen at the lower TPOAb levels (data not shown).

Using different dilutions of the 5 µg AE label did not produce any improvement in assay results, with increased imprecision evident with increased AE concentration. The 'high-dose hook' effect (with the paradoxical decline in RLUs at the higher TPOAb concentrations) was demonstrated at an 'L4' control dilution of 800-fold, but results were slightly obscured by imprecision [Fig 32]. The 'high-dose' hook effect was again illustrated at an 'L4' control

dilution ratio of 1:800 and 1:400 with the magnetic particles diluted 1:20 and 1:10 respectively [Fig 33]. These results were further endorsed by the experiment [see 3.3.3] which demonstrated that the magnetic particles (diluted 1:20) were rate-limiting with the high 'L4' control diluted 400-fold [see Table 2: 3.3.3].

The Chemiluminescent Immunoassay [2.7: Method 3 (System 2)]

This assay involved the labelling of the anti-TPO antibody, resulting in a competitive assay, in which the RLUs were inversely proportional to TPOAb, in contrast to Method 3 (System 1) which involved labelling the TPO antigen with AE, resulting in a non-competitive immunoassay. This proved to be the least viable of the chemiluminescent assays and produced similar results to the enzyme-immunometric assay [3.2], with very little difference being shown between consecutive standard absorbances [Fig 38]. A series of 'L4' dilutions which provided a wide range of TPOAb titres were also assayed with various dilutions of the Biotin-TPO label (from 10-fold to 1000-fold) and the Acrid-IgG label diluted both 1:1000 and 1:2000, but still no improvement was demonstrated (data not shown). The further purification of the Acrid-IgG label [2.7.6] resulted in no uptake of RLUs by the standards at all [Fig 44]. The results perhaps, reflecting the increased biochemical complexity of this method.

The Solid-Phase Coated-Tube Assay [2.8: Method 3 (System 3)]

This sandwich assay mimicked the ELISA, with the solubilised TPO antigen immobilised onto plastic tubes. A commercial preparation of sheep anti-human IgG labelled with AE, complexed with the TPOAb (attached to the immobilised TPO) providing a direct quantitation of the TPOAb.

The results indicated a considerable improvement in response as compared with Method 3: Systems 1 & 2, with a very good correlation of results with the ELISA and indirect agglutination (as discussed later). The logarithmically transformed assay data, was graphically represented [Fig 46]. The new Acrid-Anti-IgG label diluted 1:1000 provided a slightly improved response, as

compared with a dilution of 1:2000 and a good response was still produced after reducing the final incubation (with label) to 30 minutes. However, a lower uptake of RLUs was evident in both these experiments, which could compromise assay sensitivity [Figs 47 & 48]. Thus, optimal assay conditions consisted of an intial 2 hour incubation for the interaction for the sample TPOAb and the immobilised TPO, followed by a 1 hour incubation with the Acrid-Anti-IgG, diluted 1:1000 [Figs 45 & 46]. The 'high-dose' hook effect was demonstrated at an 'L4' control dilution of 200-fold (approximately 172 kIU/L) [Fig 49], which exceeded that shown in Method 3 (System 1) [Fig 33]. However, the threshold was not as high as that shown with the ELISA, which did not exhibit the effect, even at a 'L4' dilution of 100-fold [Fig 50].

The enzyme-immunometric assay (Method 2) and the chemiluminometric assay [Method 3 (System 1)] did not produce the same successful response as exhibited with the IRMA (Method 1). As the Protein A suspension had been successfully applied to the IRMA, this inferred that it should not be the dysfunctional assay component. This indicated the source of the problem as being the HRP-TPO label. Similarly, with Method 3 (System 1) the magnetic particles coupled to anti-human IgG had been used in a similar assay system to measure TgAb [Appendix 5], which produced a far superior assay (with an uptake of 17% of AE for the top standard), which implicated the source of the problem as being the Acrid-TPO label. However, these findings warrant further investigation, as they are based on the assumption that both of the magnetic solid-phases used, had an equal affinity and avidity for IgG, but an actual direct comparison of each, in each of the assay systems had not been made.

A modest uptake of 22% of the initial HRP activity by the TPO was demonstrated. However, the experiment [see 3.2.1] indicated that HRP activity was present in various dilutions of the HRP-TPO label when incubated with the OPD substrate [Fig 22]. The elution profile of the Acrid-TPO label demonstrated a high uptake of AE [Fig 24] and similarly, a >50% uptake of biotin was also demonstrated upon the biotinylation of TPO [Fig 34], which indicated that TPO could be successfully labelled with both AE and biotin.

Further Discussion of Assay Components and their Viability

(i) The Size of the TPO Molecule

The Tg molecule being a larger molecule of 660,000 kDa (Williams & Goodburn, 1983, Premawardhana *et al.*, 1994) as compared to TPO of average 103-117 kDa (Banga *et al.*, 1984; O'Connor & Davies, 1990; Banga *et al.*, 1991; Weetman & McGregor, 1994) had a greater uptake of AE upon labelling, which would afford greater assay sensitivity. The top standard in the Tg assay (using Acrid-Tg) [Appendix 6] had an uptake of AE with RLUs in the order of 10^6 as compared with 10^4 for the equivalent TPO standard. As TPO is a smaller molecule than Tg, it perhaps has less surface area for the interaction with the AE, with steric hindrance possibly preventing the appropriate interaction with the anti-human IgG labelled magnetic particles. However, the commercial [^{125}I]-labelled TPO proved to be functional in the IRMA (Method 1), which indicated that TPO could be effectively radiolabelled and used in a similar assay using a magnetic antibody suspension.

(ii) Loss or change in TPO Antigenicity upon Labelling

A possible explanation for the lack of response in the assays, could be a loss or modification in the antigenicity of the TPO (perhaps associated with steric hindrance) after coupling to the AE or biotin label. No methodology was currently available to investigate whether the TPO had retained its original antigenic properties after labelling with HRP, but the other TPO labels (i.e. Acrid-TPO and Biotin-TPO) were investigated for any change in their antigenic response as compared with the unlabelled, microsomal preparation.

An initial experiment involved adding increasing amounts of the TPO label (diluted in a high TPOAb titre sample) across an ELISA plate [see 2.6.5 & 2.7.3 (i)] and the response compared with the unlabelled TPO and the PBS blank. The TPO in the sample added, would compete with the solubilised TPO coated on the plate for the TPOAb (present in the patient serum used to dilute the TPO). The more active the TPO added, the greater would be the competition for the TPOAb, therefore less TPOAb will bind to the coated well, resulting in a decrease in absorbance at 410 nm with increasing TPO activity

added. The results showed the same decrease in absorbance at 410 nm with the crude TPO and the labelled TPO, indicating that there did not appear to be a loss of antigenicity of the TPO after labelling with the AE [Fig 25] or biotin [Fig 35].

However, an alternative experiment [3.4.1: Experiment 2] in which the Biotin-TPO was immobilised onto an ELISA microtitre plate, indicated that although the antigenic properties had not been lost, a subtle modification in the antigenicity of the TPO had manifested upon biotinylation. The Biotin-TPO label produced a good standard curve response [Fig 36], however, the Biotin-TPO produced higher blank values than its unlabelled counterpart, which indicated increased non-specific binding and also produced an erroneously high result for a 'Normal' control, again not demonstrated in unlabelled TPO.

The erroneous 'Normal' result was also produced in the coated-tube assay, using Biotin-TPO as the coating-antigen [Experiment 3: 3.4.1], and in another experiment [Experiment 3: 3.4.2] in which the peroxidase conjugate/substrate system was used instead of measuring the RLUs of the AE attached to the final magnetic pellet. This acquired cross-reactivity indicated a modification in the antigenic properties of the TPO upon biotinylation, not revealed in the initial antigenicity check.

The ECL protein biotinylation system has been designed to label primary amino groups, including enzymes. The biotin ester used in the ECL kit has been synthesised incorporating a 'spacer arm' which ensures that the protein linked biotin is freely accessible to bind streptavidin. The incorporation of a 'spacer arm' should overcome the associated problems of steric hindrance and possibly result in a more stable streptavidin-biotin complex.

Although the biotinylation of a protein is a relatively mild chemical reaction, the number and location of biotin-moieties coupled to the amino groups of a protein can alter its physico-chemical properties and impair biological activity (Miralles *et al.*, 1991; Higashi *et al.*, 1992; Jokiranta & Meri, 1993; Muzykantov *et al.*, 1995). A remarkable increase in hydrophobicity of the biotinylated protein has been demonstrated as compared with its unlabelled counterpart by Storm *et al.*, (1996) who recommend a biotin optimum for a given protein, in

order to yield a high detection signal without loss of biological function. In this study 40 µl of biotin solution (concentration not stated) per 1 mg of protein was used as defined by ECL.

(iii) The Purity of the TPO

In addition to TPO being a smaller molecule than Tg, it is a more complex transmembrane protein (Banga *et al.*, 1991; McLachlan & Rapoport, 1995), which has proved to have been difficult to obtain free of contamination of serum components such as Tg (Ruf *et al.*, 1988, Feldt-Rasmussen, 1996). The less pure microsomal TPO preparation (and labelled counterpart) was successfully applied and proved to be viable in the ELISA and coated-tube assay, but was proved to be less viable in the enzyme-immunometric and chemiluminometric assays, which strongly implicated the purity of the TPO as being the source of the problem. The Tg and TPO used in this study were produced at different stages within the same preparation procedure (Groves *et al.*, 1990). The purity of the Tg, however, was sufficient for use in both Method 3 (System 1) and the ELISA (Groves *et al.*, 1990), whereas the TPO, although of adequate purity for use in the ELISA (Groves *et al.*, 1990), possibly lacked the necessary purity for the more random assay system [Method 3 (System 1)].

The label used in the IRMA was a commercial preparation of TPO labelled with [^{125}I], which guaranteed a pure source of TPO antigen, which probably accounted for the higher sensitivity and specificity demonstrated. The label was produced from the same source as the [^{125}I] label used by Beever *et al.*, 1989 which consisted of an 'in-house' preparation of labelled TPO, obtained from the microsomal fraction of homogenised thyroid tissue (prepared by differential centrifugation similar to the procedure described by Groves *et al.* (1990). Beever *et al.* (1989) used a further purified TPO preparation (which included the use of affinity chromatography) labelled with [^{125}I], which resulted in an assay of high sensitivity and specificity. Thus, the TPO used in the non-radioactive methods, in this project's assays, appeared to be of insufficient purity, which introduced a high degree of non-specific binding caused by other

interfering species [such as thyroglobulin (Tg)] which had also been labelled together with only a small percentage of TPO.

As TPO does not lend itself favourably to an effective purification procedure (Ruf *et al.*, 1988) being time-consuming, and both technically and biologically difficult, and not amenable to the development of a robust assay for routine use, it was not attempted within the present project. The preparation of recombinant TPO is again a very lengthy and involved procedure and was not a viable option in this study, and in any event even if a source was available there are theoretical reasons why recombinant TPO might not be suitable. Sera containing human thyroid autoantibodies display a variety of antigen-specific immunoglobulins of different classes and subclasses with different affinity and avidity in their epitope reaction (Feldt-Rasmussen, 1996). Reports have been made of up to six (Hamada *et al.*, 1987; Weetman *et al.*, 1987; Doble *et al.*, 1988; Banga *et al.*, 1991) or seven epitopes (Ruf *et al.*, 1989), with some patients having autoantibodies raised against more than one of the epitopes (Yokoyama *et al.*, 1989). This imposes a problem on deciding upon which section/epitope of the TPO molecule should act as the target for the TPOAb, against which the recombinant TPO can be raised. The ideal label would be able to encompass all the possible epitopes for interaction, so as to measure all the possible autoantibodies which may be present in the human population. Therefore, because of the varying antigenic sites on the TPO molecule investigators must decide which epitope(s) of TPO antigen are the most important in the autoantibody reaction, [with a sequence C2 thought to harbour a major epitope (Ludgate *et al.*, 1989)]. Attempts have been made to produce recombinant TPO of both full length and truncated forms (Feldt-Rasmussen, 1996).

However, the ELISA and the coated-tube assay, used in this study, also cannot guarantee the identification of all the various epitopes, as the different thyroid tissue used to prepare the TPO can confer immunological variation upon the assay system, with even slight differences in its preparation capable of affecting its performance.

The Purified IgG (containing Anti-TPO Antibody): Used in Method 3 (System 2)

The Biotin-TPO label appeared to be functional in the coated-tube assay Method 3 (System 3) [2.7] and in the ELISA [Appendix 2:7], but its viability in Method 3 (System 2) was questionable. However, experiments indicated that the lack of response (using the biotin-streptavidin interaction) could also be attributed to a lack of purity of the IgG labelled with AE.

The results of Experiment 1 [described in 3.4.2] confirmed the efficacy of the Acrid-IgG-Biotin-TPO-Streptavidin magnetic bead interaction, with the demonstration of a proportional uptake of AE with increased Acrid-IgG label added. However, graphical representation of the final RLUs indicated that the addition of a sample with a high TPOAb titre to a second set of tubes appeared to have no effect on the amount of Acrid-IgG label being taken up by the beads [Fig 39], inferring that the Acrid-IgG label was not competing effectively with the high TPOAb sample for the Biotin-TPO label. The TPOAb in the sample should compete with the Acrid-IgG label, which would result in less Acrid-IgG label being bound to the Biotin-TPO label and subsequently less RLUs bound to the streptavidin beads, which was not shown.

The streptavidin beads were demonstrated as being functional as described in Experiment 2 [3.4.2]. Supernatant obtained from incubating the Biotin-TPO label with streptavidin beads and subsequently diluted in a sample containing a high TPOAb titre, was added in increasing amounts across an ELISA plate. Any TPO present remaining in the supernatant, would have competed with the immobilised TPO, resulting in less TPOAb binding on the plate, producing a decreased absorbance with increasing TPO concentration added. As there was no difference in response between the label and the blank, this strongly suggested that the Biotin-TPO label had been effectively removed from the supernatant by the streptavidin beads (data not shown).

The results of Experiment 3 [3.4.2], (in which the final Biotin-TPO-Streptavidin-Acrid-IgG/TPOAb complex was quantitated using the ELISA peroxidase conjugate/substrate reaction), inferred that the Biotin-TPO was functional and had reacted proportionately with the standards and in turn with

the beads, as indicated by the final supernatants (absorbance measured at 410 nm) [Fig 40]. This experiment endorsed the findings that the Acrid-IgG label was not competing effectively with the sample TPOAb for the Biotin-TPO label.

As the acridinium ester is designed to couple with the IgG protein component, the actual labelling procedure should not be a problem, thus implicating the purity of the original anti-TPO as the source of the problem, which resulted in the ineffective competition with the sample TPOAb for the Biotin-TPO label. It could be surmised that perhaps other non anti-TPO immunoglobulins had been labelled with the AE and only a very small amount of IgG specific to TPO was actually labelled. Even further purification by affinity chromatography did not apparently produce sufficient yield. However, perhaps the actual system was not viable due to its complexity both biochemically and technically.

The results of the project indicated that the crude TPO functioned successfully in the solid-phase assay systems (i.e. the coated-tube assay and the ELISA), which involved the immobilisation of the TPO antigen onto the solid support. The crude preparation (although apparently retaining its antigenicity after labelling) was less effective in the assays using magnetic particles to effect the separation of the labelled autoantigen-autoantibody complexes. This was apparent with the Biotin-TPO label, which produced a reasonable response when applied to the ELISA and the coated-tube assay, but not in the complex TPO-Biotin-Streptavidin system. This inferred that the former solid-phase assays did not rely on as high a degree of purity of TPO, and would account for the success of the IRMA (Method 1) which used a commercial radioactive label and hence, a purified TPO preparation.

The systems which utilised the magnetic particles (in homogeneous solution), although providing increased surface area for interaction (which should increase assay sensitivity), probably introduced a greater degree of variability in the exposure of the various antigenic sites on the TPO molecule. This variability is reduced in the solid-phase assays, as the TPO is immobilised onto the plastic surface, which serves to decrease the randomness of the exposed sites for interaction with the TPOAb.

The Coated-Tube Assay

Assay systems such as agglutination, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) can give useful clinical information, but they all depend on the indirect measurement of the interaction between autoantibody and autoantigen, and this can result in limitations of sensitivity, precision and ease of handling. The coated-tube assay provided a direct measurement of TPOAb, with decreased reagent manipulation as compared with the ELISA. However, the coated-tube assay system (with analyses set up in duplicate), although perhaps involving less stages, did not provide any other immediate advantage over the established ELISA assay (analysed in quadruplicate). Both the assays required special, dedicated, measuring systems (i.e. ELISA requires a plate reader and the coated-tube assay requires the Ciba Corning [now Bayer] Magic® Lite Luminometer). The ELISA proved to be less technically cumbersome, with the micro-titre plates easier to wash than the tubes, and problems were encountered on occasions with the ineffective draining of the tubes, which could account for the lack of precision evident on some assays. However, plate-readers are now comm ercially available which are capable of measuring luminescence in micro-titre plates (such as the 'Orion' microplate luminometer by Flowgen). Both assay systems can be semi-automated with the application of sample processors and automated wash systems.

The AE label demonstrated a long shelf-life and was still functional after six months, which provided a significant advantage over the use of radioactive reagents, also requiring no stringent storage or disposal procedures. It was noted in the latter stages of the study that the assay RLUs had decreased in the coated-tube assay as compared with the start of the study. There also appeared to be a greater degree of imprecision in the duplicate analyses (especially at high TPOAb levels), which would warrant further investigation. This could be accounted for by reagent deterioration, however, technical problems were also encountered with the Magic® Lite Luminometer, which could also have introduced erroneus results.

Statistical Analysis of Data

Comparison of the Coated-Tube Assay and ELISA

Among the tests that could be applied to describe the relationship between results by two methods are the least-squares linear regression, Pearson correlation, Deming regression analysis and the Passing and Bablok's method (Jones & Payne, 1997).

The Least-Squares Linear Regression

The least-squares linear regression is applied to paired measurements with the object of fitting the best straight line which will pass through the plotted points to predict the average value of any one variable, the dependent variable (usually named y), to be expected at any value of the other independent variable (usually named x). The assumptions of the parametric test for association in least squares linear regression, are that there is a straight line which can be drawn through the means of the x at any y value in such a way that the data points have a 'Normal Distribution' about it in the y direction at each value of x and that the S.D.s of y are similar at all values of x (Jones & Payne, 1997).

Non-parametric tests should be used when the data is not normally distributed, however, the logarithmic transformation of the data for the coated-tube assay and ELISA allowed the processing of the parametric 'scatter' plot or the 'least squares regression analysis'. The correlation coefficient (r) derived from comparing the coated-tube assay and the ELISA was 0.96, which indicated a very close correlation between the methods, with a probability $P = <0.001$ [Fig 51].

The disadvantage of using the least-squares regression to describe the relation between two methods is the assumption that the independent variable is determined without error, however, in method comparison both variables are measured with error. The correlation coefficient (r) (introduced by Karl Pearson) is a numerical way of expressing how closely the slopes of the two regression lines agree. The Pearson correlation coefficient gives no

information of value in method comparison studies, because (r) can be highly significant when there is no obvious bias between the two methods. A correlation coefficient (r) near one will be obtained when the data points lie close to a straight line even when the slope of the line is very different from one. It measures the strength of association and not of agreement (Jones & Payne, 1997).

Deming Method

The data were also processed using the Deming Plot, which adopted the approach of Kummel (1879) to solve the problem of describing the relationship between variables both measured with error, by proposing that the sum of the squares of the deviation from the line should be minimised in both the x and y directions at the same time, taking account of the analytical imprecision of each method, not done in the least-squares linear regression.

The major advantage of the Deming's method is that it gives only a single regression line whether x or y is used as the 'independent' variable. The intercept is calculated as in conventional least squares regression as the mean of y minus the product of the slope and the mean of x or vice versa. The calculation provided the standard error (SE) of the slope (0.029), and of the intercept (3.46) (in the y direction if x is taken as the independent variable), so that the line can be plotted with confidence limits [Fig 52 (i)].

The disadvantage of the Deming method is that it assumes the ratio of the analytical variances of the two methods is constant over the range of measurements. Thus, the method maybe in error if the analytical S.D.s increase with measured values at different rates. Like the least-squares linear regression, Deming's method is greatly influenced by data pairs that have substantially higher or lower values than the bulk of the results (Jones & Payne, 1997).

The Deming plot [Fig 52 (i)] again demonstrated a good correlation between the coated-tube assay and ELISA, with the residuals demonstrating increased variability at the higher TPOAb assay range [Fig 52 (ii)]. The residual value is

the term used to denote the distance between an observed value and the value given by the regression line known as the 'fitted' value (Altman, 1991).

Passing and Bablok Model

The data were also analysed using the 'Passing & Bablok' Model (a modification of Thiel's procedure adapted to method comparison studies) [Figs 51 (i) & (ii)]. Essentially, the slopes of all the straight lines between any two points are used to calculate the slope. Like Deming's method, it gives only one regression line. The method has been well publicised in the field of clinical chemistry, but has not been extensively used because the method of calculation has not been widely available.

The method introduces a median shift which, unlike the Deming method, has no bias in its estimates of slope and intercept when measurement error increases with concentration at different rates. The method has the additional advantage that each pair of results is given equal weight in the calculation so that, unlike Deming's method, it is not unduly biased by extreme values. It has the disadvantage that it gives no measure of scatter (Jones & Payne, 1997).

A good comparison was again demonstrated between the coated-tube assay and the ELISA, by the fact that the fitted regression line is similar to the identity line [Fig 52 (i)]. The cumulative sum (cusum) chart [Fig 52 (ii)] shows the cumulative count of residuals above and below the regression line and is used to determine linearity. Large peaks or troughs indicate many residuals on the same side of the regression line and possible non-linearity. The results demonstrated, that there was less agreement between the assays at the higher TPOAb range, by the sudden trough exhibited between running numbers 80 and 100 [Fig 52 (ii)].

Comparison of Indirect Agglutination with the Coated-Tube Assay and ELISA

A comparison was also carried out for 37 samples between indirect agglutination and the coated-tube assay and ELISA [Figs 54 & 55 respectively]. As the data was non-parametric, and the data for the indirect

agglutination had to be rank-ordered, the Wilcoxon Mann-Whitney Ranked Statistical test was applied to investigate the differences between two independent samples. This test is the non-parametric equivalent of the paired 't' test, and assigns the results in each category in ascending numerical values, and is used to test the null hypothesis that the distribution of two variables are the same (Jones & Payne, 1997). In this study the data obtained in the indirect agglutination assay was ranked from 1 to 8, which represented the result titres from negative to 1:500,000. The ELISA and coated-tube assay data was logarithmically transformed for the statistical comparison. A good correlation was demonstrated between the coated tube and indirect agglutination ($r = 0.80$, $P = <0.001$, $n = 37$) and also with ELISA and indirect agglutination ($r = 0.85$, $P = <0.001$, $n = 37$).

For the comparison of quantitative methods, measurements should be made in duplicate as in the coated-tube assay (with the ELISA performed in quadruplicate). This allows the calculation of the imprecision of each method at a range of levels and the assessment of whether outliers are due to imprecision (when the duplicates are likely to differ widely) or to a difference in specificity or susceptibilty to analytical interference between methods (when the duplicates are likely to give similar values). The outliers in the coated-tube assay were produced predominantly due to the imprecision of measurements, suggestive of a processing problem rather than an intrinsic methodological problem. The imprecision was more evident at higher TPOAb activites and towards the end of the study, possibly attributable to reagent deterioration. Erroneous values were produced in the ELISA, but to a lesser extent than in the coated-tube assay and quadruplicate analysis tended to reduce repeat analysis. However, another possible source of variation could be produced by the difference in the antigenic determinants intrinsic in the TPO antigen itself and its tissue source, and also due to a lack in specificity associated with its degree of purity.

Precision Studies, Analytical Specificity and Sensitivity

Precision studies indicated comparable data for the coated-tube assay and ELISA, with perhaps an improved 'within-run' precision seen with the ELISA [Tables 3 & 4: 3.5.3].

The 'detection limit' (analytical sensitivity) (defined as the smallest value that can be distinguished from zero with a defined degree of confidence) was found to be 2.46 kIU/L which did not provide the level of sensitivity offered with the IRMA, nor that obtained with ELISA (0.8 kIU/L). This value is determined by the degree of non-specific binding associated with the purity of the TPO coating antigen.

Studies to determine the 'analytical specificity' (described as the extent to which substances which maybe present in a sample interfere with the analysis) indicated that smooth muscle antibodies (SMA) did not interfere with the coated-tube assay, but did exhibit positive interference in the ELISA, however, this could be attributed to the icteric nature of the sample which would interfere with the colorimetric measurement. Morita *et al.* (1995) also reported SMA interference with the earlier immunofixation (IMF) techniques. Positive TPOAb results were produced in the coated-tube assay and in the ELISA on samples positive for mitochondrial antibody (MIT) and parietal cell antibody (PCA). However, the results were invalidated due to the demonstration of incidental positivity for TPOAb when assayed by indirect agglutination. No interference was indicated with a range of rheumatoid factor (RF) titres in either the coated-tube assay or the ELISA.

The coated-tube assay proved to be superior to the ELISA and the Cambridge Life Sciences 'Autozyme™' kit for measuring TPOAb in serum in that haemolysed, lipaemic and icteric samples did not produce any false positive (erroneous) values in the coated-tube assay. The ELISA demonstrated positive interference with the icteric sample and the manufacturer's of the 'Autozyme™' kit advise against the use of the latter three sample interferences.

Determination of the Assay 'Cut-off' Value for 'Normal' versus 'Abnormal' Results

A first line screening test should detect all disease positive cases and should give no false negatives. On the other hand, a test used to confirm a diagnosis should be maximally 'specific' and should not misclassify patients as positive when they do not have the disease. In addition, a test used for monitoring treatment or disease needs to be highly reproducible.

Various statistical methods can be applied to the determination of the 'cut-off' value. Groves *et al.* (1990) used a non-parametric method, as upon carrying out a 'Normal probability plot' their data were skewed and the 'cut-off' was applied manually to determine the central 95% fraction. Intuitive assessment can provide a rule of thumb guidance as to the expected upper and lower limits of a particular value, but is not recommended (Jones & Payne, 1997). When using the 95% percentile for healthy individuals, greatly variable values have been obtained, partly attributable to the selected subject population (i.e. differences with the age and gender ratio), differences in TPOAb affinities in different serum samples, the method used for TPOAb measurement [including differences associated with the dilution media employed (Feldt-Rasmussen, 1996)], and also the final reporting of results.

Assessment of a reference range for TPOAb in a normal population is a controversial issue, mainly due to the fact that TPOAb, as well as other thyroid autoantibodies may be positive in a healthy person with completely normal thyroid function. It is a matter of much speculation as to whether the presence of low concentrations of TPOAb is indicative of false-positive measurements or may indicate individuals with a predisposition to disease. Additionally, not all of these individuals progress to develop disease and there is no way of identifying which individuals will, as a complex interplay of genetic and environmental factors is involved.

A useful technique advocated for determining the optimum 'cut-off' level is the Receiver Operator Characteristic (ROC) curve analysis. It can be applied to clinical measurements in order to identify optimum test 'cut-off' values (Jones & Payne, 1997) and its usefulness as a fundamental evaluation tool in clinical

medicine was reviewed by Zweig & Campbell (1993). The test is based on a graphical representation of sensitivity (y-axis) plotted against 1 minus specificity (x-axis) for a range of threshold values. The trade-off between sensitivity and specificity can be appreciated visually, or the test may be extended by comparing derived values from the curves, including the areas under the curves. This analysis was not attempted in this study as access to all the required clinical data was not possible to associate the diseased state with the measured TPOAb level.

The determination of the 'cut-off' value (to distinguish between 'normal' and 'abnormal') to screen for subclinical AITD was not determined in this study, as to provide valid results a large population study would be required of apparently 'normal' individuals, as carried out by Groves *et al.* (1990), who studied 832 blood donor samples. However, as a good correlation of results was evident for the coated-tube assay and the ELISA, the 'cut-off' value of 19.6 kIU/L used for the ELISA was applied to the coated-tube assay and only one discordant result was indicated (which was borderline for both methods) and would warrant further investigation in both cases.

The clinical sensitivity and specificity for the coated-tube assay (n=100) was calculated as being 100% and 95% respectively, using the 'cut-off' value of 19.6 kIU/L for both ELISA and the coated-tube assay (assuming the ELISA to be the 'gold standard' [Table 6: 3.5.5 (i)]. A sensitivity of 73% and a specificity of 100% was calculated for indirect agglutination (n=37), using the 'cut-off' value of 19.6 kIU/L in the coated-tube assay and a titre of 1:100 as being negative in indirect agglutination [Table 7: 3.5.5 (ii)]. The limited data indicated that indirect agglutination tended to underestimate at high TPOAb concentrations and also be less sensitive at lower TPOAb concentrations, as compared with the coated-tube assay and ELISA.

The field of thyroid autoantibody testing has been hampered by poor attempts at interlaboratory or international standardisation of the methods used in clinical practice. It is therefore difficult to compare the analytical sensitivity, accuracy and precision profiles of the various methods presented in the literature or by commercial companies. Thus, although there is a reference

preparation 66/387, the results are also dependent on the method principle, with results being quantified in terms of immunoglobulin content, antigen/epitope reactivity or binding capacity (Feldt-Rasmussen, 1996). Additionally the various studies in the literature involve the use of purified human TPO, porcine TPO, and more recently recombinant TPO or truncated recombinant TPO, with some method principles (e.g. competitive RIA) being highly dependent on a very pure source of TPO, whereas some methods are less influenced by impurites, the latter endorsed in this study.

Screening for Subclinical Autoimmune Thyroid Disease

A screening test for a particular condition must meet certain criteria before implementation, and be both clinically and financially viable, satisfying the requirements of sensitivity and specificity. It should be easy to perform and cause a minimum of stress to the individuals concerned, with an appropriate and effective treatment regime subsequently available.

A screening test to detect thyroid peroxidase autoantibodies in asymptomatic individuals in 'high-risk' groups could possibly provide intervention at an earlier stage of the disease process, which could preclude the need for lifelong supplementation of thyroid hormones or surgical intervention, which in itself is a substantial drain on health resources, or avoid the devastating consequences sometimes associated with PPT (Burrow, 1993; Pop *et al.*, 1995).

The presentation of an unexplained increased, isolated thyroid-stimulating hormone (TSH) can indicate the presence of subclinical hypothyroidism or hyperthyroidism (Weetman, 1997), and provides a valuable 'first-line' investigation, which can be followed-up with the more specific and expensive TPOAb assay. The population prevalence of subclinical hypothyroidism (i.e. isolated raised TSH) varies between studies, with a range of approximately 1.4 - 7.8% (Weetman, 1997). The prevalence of asymptomatic autoimmune thyroid disease in apparently normal individuals (by measuring thyroid autoantibodies) ranges from 3.6 - 8.4% (Roti *et al.*, 1992; Sundbeck *et al.*, 1995) to 15% (Weetman & McGregor 1992), reflecting the chronological

increase in methodological sensitivity. Women are around 3 times more frequently affected than men, especially in elderly women (Weetman, 2000). Women with both a raised TSH and positive thyroid autoantibodies are at a greater risk of developing overt hypothyroidism than with either an isolated increased TSH or TPOAb, with the relative risks even higher in men (Weetman, 2000).

The screening of the general population for subclinical hypothyroidism using TSH measurement is not advocated (Helfand, 1990; Vanderpump *et al.*, 1996). Similarly, the general screening for subclinical AITD using TPOAb measurement is not considered to be a viable option, as not all TPOAb positive individuals progress to develop disease, but it is recommended that 'high-risk' groups are targeted for cost effectiveness. The annual risk of developing hypothyroidism in women is 4.3% when both anti-thyroid antibodies (namely TPOAb) are present and serum TSH is raised. If a raised serum TSH alone is found the annual risk of developing hypothyroidism is 2.6% and the presence of anti-thyroid antibodies or raised serum TSH alone was associated with a highly significantly raised risk (5% per year) of developing hypothyroidism at twenty years (Vanderpump *et al.*, 1995). The study by Vanderpump *et al.* (1995) has important implications for the recommendation of treatment of asymptomatic women with markers of thyroid disease, i.e. subclinical hypothyroidism. In addition, the appearance of thyroid autoantibodies seemed to be coincident with the menopause (Vanderpump *et al.*, 1995). Thus, focusing on women over 40 years with non-specific symptoms would be a favourable approach (Weetman, 1997), and also on patients taking lithium or amiodarone who are also at risk of developing hypothyroidism and thyrotoxicosis and require regular monitoring of thyroid function (Vanderpump *et al.*, 199).

Weetman, (2000) still does not advocate the general screening of pregnant women for subclinical AITD by measuring TPOAb, as only 50% of females who are TPOAb positive proceed to develop disease (Weetman, 1994). Targeting the more vulnerable or 'high-risk' sections of the population i.e. young females, especially those with Type 1 Diabetes Mellitus (Bech, *et al.*,

1991) or other autoimmune endocrine disorders (Weetman, 1994) and individuals with a family history of disease, would prove to be more beneficial. It is recommended that women who are Type 1 Diabetes Mellitus antepartum should have thyroid antibody measurements in the first trimester, with careful follow up of those with positive results. Also, any women who develops PPT should be offered annual follow-up as about a quarter of these women will develop overt hypothyroidism within the next five years (Othman *et al.*, 1990). This approach is favoured by Weetman, (2000) who believes that the general screening and monitoring of TPOAb during pregnancy would result in further unwanted stress and anxiety, with no obvious advantage being gained.

The advent of new technology may prove that the more specific TPOAb test will become more cost-effective and be equally available for routine use as TSH measurements. The newer automated systems (with fully robotic systems also becoming available) are now capable of measuring chemical tests effectively and quickly. Bayer/Chiron are currently developing a method for measuring TPOAb which utilises acridinium ester and magnetic particles as the separation phase. Methods are also being developed for the measurement of TPOAb by Abbott on the 'AxSYM' Analyser, which utilises microparticle enhanced immunoassay (MEIA), and also by Johnson & Johnson on the 'Vitros E' Analyser which utilises solid-phase enzyme-immunoassay.

Treatment

As previously stated, before a screening programme can be introduced, an effective treatment regime must also be available, but controversy exists as to the appropriate and effective use of current treatments. An argument in favour of instituting thyroxine replacement is that it prevents the onset of overt hypothyroidism. This is particularly persuasive for people with raised thyroid stimulating hormone concentrations plus thyroid antibodies, who have an annual risk of developing overt hypothyroidism of around 5% (Vanderpump *et al.*, 1995). The detection of subclinical hypothyroidism (using a sensitive TSH assay) warrants further testing for thyroid peroxidase autoantibodies and if these are positive there is now general consensus that treatment with

thyroxine is indicated (Weetman, 2000). For those individuals with subclinical hypothyroidism but negative antibodies, the need for treatment is yet to be established, although many endocrinologists would give a trial of thyroxine, as providing the TSH concentrations are restored to the reference range and properly monitored, no adverse effects have been indicated (Weetman, 1997).

There are modest benefits from treating subclinical hypothyroidism (Weetman, 1997). Trials of treatment in sub-clinical hypothyroidism have shown small but significant benefits in symptom scores and psychometric performance, and there may be mild benefits with regard to lowering of cholesterol, as subclinical hypothyroidism is 2-3 times more common than expected in people with increased total plasma cholesterol concentrations (Weetman, 2000).

Reports have been made, whereby asymptomatic patients with autoantibodies, who are the treated with thyroxine can revert to normality, with no apparent recurrence of disease. Nystrom *et al.* (1988) have shown that asymptomatic female patients with subclinical hypothyroidism responded positively to therapy, as shown in a controlled trial in which the administration of L-thyroxine stopped some individuals from proceding onto developing autoimmune disease.

Hidaka *et al.* (1994) state that it may be possible that early diagnosis and the immediate start of appropriate drug therapy will bring a prompt remission in Graves' thyrotoxicosis, but no large-scale investigation had been carried out to substantiate their claims. Treatment of Graves' disease with antithyroid drugs and of idiopathic myxoedema or hypothyroid Hashimoto's thyroiditis with L-T4 was also associated with a significant decrease in TPOAb (Jansson *et al.*, 1985; Mariotti *et al.*, 1990; Rieu *et al.*, 1994; Takasu *et al.*, 1990; Takasu *et al.*, 1992). In addition, Hashizume *et al.*, 1991 and Rieu *et al.*, 1994 agreed that thyroid hormonal status (changed with appropriate treatment) can modulate thyroid autoimmunity expression.

However, conflicting results have been reported on the effect of treatment on euthyroid individuals (Rieu *et al.*, 1994). A decrease in mean levels of TPOAb in response to L-T4 therapy in euthyroid patients indicated by Rieu *et al.* (1994) showed discrepancies with other studies (Chiovata *et al.*, 1986; Takasu

et al., 1990). This could possibly be explained by the variation introduced by differences in TPOAb methodology used, study-plans and treatment regimes. Also, PPT symptoms of depression are more common in women who have thyroid antibodies than in those without, irrespective of biochemical thyroid dysfunction. The implication being that some mood disturbance in subclinical hypothyroidism has an immunological rather than endocrinological basis, in which thyroxine treatment would not help (Weetman, 1997).

The cloning and expression of TPO as a recombinant protein has allowed progress on the characterisation of the autoantigenic epitopes recognised by autoantibodies. The main immunogenic region on the molecule recognised by autoantibodies has been localised to the carboxyl terminal. The availability of recombinant autoantigen, together with synthetic peptides derived from the amino acid sequence of TPO, will now facilitate the characteristics of T cell populations and the epitopes recognised on TPO from patients with AITD. This will allow therapeutic strategies at an antigen level to be addressed (Banga *et al.*, 1991).

There are reports of immunisation procedures being developed which may serve to prevent the onset of autoimmune disease. A number of approaches aimed at restoring self-tolerance in autoimmune disease are now being implemented in clinical trials in humans (Steinman, 1995). Oral tolerance is a long recognised method to induce peripheral immune tolerance and the oral administration of autoantigens may find a place in the treatment of human organ-specific inflammatory autoimmune diseases (Weiner *et al.*, 1994). Attempts to induce tolerance by feeding antigen have given encouraging results in small trials in patients with multiple sclerosis (MS) and rheumatoid arthritis (RA) (Weiner *et al.*, 1994) and may eventually be applied to AITD. The novel macrolide antibiotic, FK-506, has been found to ameliorate human AITD lesions *in vivo* by the inhibition of human intrathyroidal lymphocyte activation which might have central role(s) in the development and progression of human AITD and could have implications for future treatment (Yoshikawa *et al.*, 1994).

Further Studies

A follow-up option would be to attempt to label a recombinant preparation of TPO with AE and use in the chemiluminometric assay [Method 3 (System 3)]. However, although a recombinant TPO label may have the apparent advantage of greater precision and sensitivity, it might not have the capacity to identify all the autoantibodies which may present, which could result in falsely negative results. It would be of interest to compare the results of the ELISA used in this study for TPOAb measurements with an ELISA system (developed by Cambridge Life Sciences), called 'Autozyme™' which utilises recombinant TPO and employs a similar conjugate/substrate system.

It would also be of interest to adapt the coated-tube assay to a micro-titre plate assay, as the final results can be quantitated using plate readers capable of measuring luminescence in micro-titre plates, which would overcome some of the disadvantages associated with using tubes.

CHAPTER 5

CONCLUSION

Chapter 5: Conclusion

Detection systems using chemiluminescent labels have been developed that are viable alternatives to radioisotopic methods. Chemiluminescence provides an extremely sensitive mode of detection, in which the reaction chemistry is typically very simple, yet very specific. The reagents require no special handling or disposal, and have excellent shelf-lives; and chemiluminescence can be detected using a wide variety of methods from simple photographic film to highly sophisticated photon counting instrumentation (e.g. the Bayer/Chiron 'Automated Chemiluminescent System'). In contrast to other non-radioactive end-points, initiation of acridinium chemiluminescence is extremely simple and robust. Quantitation is rapid (typically 2 seconds) and requires no substrate incubations or complex chemical manipulations prior to measurement.

The development of a viable chemiluminescent assay system using acridinium ester and magnetic particles as the separation medium, for the screening of TPOAb in human serum was not achieved in this study. However, the coated-tube assay, an alternative approach to the ELISA solid-phase system was devised, which produced acceptable assay data. The assay accommodated the use of the less pure TPO which could not be applied successfully to the other assay systems. The coated-tube assay was a direct method of analysis as compared to the indirect ELISA and involved less stages, but the assay time was only reduced by approximately 30 minutes. Acceptable precision was demonstrated, but the ELISA proved to be superior at the higher TPOAb levels. However, imprecision in the coated-tube assay could have been attributed to reagent deterioration in the latter stages of the study, and would merit further investigation to substantiate.

The provision of immunoassay reagents in the form of 'kits', sophisticated automated systems, and the considerable involvement of commercial interests, exemplifies the competitive nature in immunoassay development and the consequent proliferation of the methodology. However, it is apparent, that even in well-evaluated methods, problems can be encountered due to intrinsic (e.g. assay constituent changes) and extrinsic (e.g. presence of heterophilic antibodies) interferences, and these must be considered in an

attempt not to report erroneous and misleading assay results, and all confirm that immunology tends to be a very complex and elusive science.

The selective screening for subclinical thyroid disease, including AITD, has been indicated to be beneficial. The earlier intervention in the disease process and subsequent treatment has been associated with alleviating symptoms or sometimes preventing the further progression of the disease. The advent of new, novel treatment regimes and techniques, together with the considerable advances evident in the field of genetics could however, ultimately result in the eradication of diseases such as AITD in the future.

REFERENCES

References

Aliquer, C., Ruf, J., Athouel-Haron, A.M. & Caryon, P. (1989) Immunocytochemical study of localisation and traffic of thyroid peroxidase/microsomal antigen. *Autoimmunity* **3**, 113-123.

Amino, N., Hagen, S.R., Yamada, N. & Refetoff, S. (1976) Measurement of circulating thyroid microsomal antibodies by the tanned red cell haemagglutination technique: its usefulness in the diagnosis of autoimmune thyroid disease. *J. Clin. Endocrinol.*, **5**, 115-125.

Anderson, J.R., Goudie, R.B., Grey, K.G. & Timbury, G.C. (1957) Autoantibodies in Addison's disease. *Lancet,* **i**, 1123-1125.

Anderson, J.R., Goudie, R.B., & Gray, K.G. (1959) Complement-fixing autoantibody to thyroglobulin in Hashimoto's disease. *Lancet,* **(March)**, 644-647.

Banga, J.P., Pryce, G., Hammond, L. & Roitt, I. (1984) Characterisation of the human thyroid microsomal antigen involved in thyroid autoimmunity. *Biochem. Soc. Trans.,* **12**, 1118-1119.

Banga, J.P., Barnett, P.S. & McGregor, A.M. (1991) Immunological and Molecular Characteristics of the thyroid peroxidase autoantigen. *Autoimmunity,* **8**, 335-343.

Barclay, M.L., Brownlie, B.E.W., Turner, J.G. & Wells, J. E. (1994) Lithium associated thyrotoxicosis: a report of 14 cases, with statistical analysis of incidence. *Clinical Endocrinology*, **40**, 759-764.

Bech, K., Hoier-Madsen, M., Feldt-Rasmussen, U., Jenson, B.M., Molsted-Pederson, L. & Kuhl, C. (1991) Thyroid function and autoimmune manifestations in insulin-dependent diabetes mellitus during and after pregnancy. *Acta Endocrinologica*, **124**, 534-539.

Belyavin, G. (1953) *J. Hyg. Camb.,* **51**, 492. (Quoted by Anderson *et al.*, 1959).

Belyavin, G. & Trotter, W.R. (1959) Investigations of thyroid antigens reacting with Hashimoto's sera. Evidence for an antigen other than thyroglobulin. *Lancet,* **i**, **November 15**, 648-652.

Beever, K., Bradbury, J., Phillips, D., McLachlan, S.M., Pegg, C., Goral, A., Overbeck, W., Feifel, G. & Rees-Smith, B. (1989) Highly sensitive assays of autoantibodies to thyroglobulin and thyroid peroxidase. *CLIN. CHEM.,* **35:9**, 1949-1954.

Bird, T. & Stephenson, J. (1973) Evaluation of a tanned red cell technique for thyroid microsomal antibodies. *J. Clin. Pathol.,* **26**, 623-627.

Bland, G.A. (1991) 'Regression' in Practical Statistics For Medical Research, 1st Edn., p. 301, Chapman & Hall, London.

Boland, J., Carey, G. & Kwiatkowski, M. (1990) The Ciba Corning ACS™ Benchtop Immunoassay Analyser. *CLIN. CHEM.*, **36:9**, 1598-1601.

Bottomley, S.P., Sutton, B.J. & Gore, M.G. (1995) Elution of human IgG from affinity columns containing immobilised variants of Protein A. *J. Immunol. Meth.*, **182**, 185-192.

Burrow, G,N. (1993) Thyroid function and hyperfunction during gestation. *Endocrine Reviews,* **14:2**, 194-202.

Calabrese, J.R., Gulledge, A.D., Hahn, K., Skewerer, R., Kotz, M., Schumacher, O.P., Gupta, M.K., Krupp, N. & Gold, P.W. (1985) Autoimmune thyroiditis in manic-depressive patients treated with lithium. *American Journal of Psychiatry*, **142**, 1318-1321.

Cambridge Life Sciences plc. 'Autozyme™ TAB'. (July 1996) Anti-Tg and Anti-TPO antibodies. (Kit Information insert).

Cavan, D.A., Penny, M.A., Jacobs, K.H., Kelly, M.A., Jenkins, D., Mijovic, C., Chow, C., Cochram, C.S., Hawkins, B.R. & Barnett, A.H. (1994) The HLA association with Graves' disease is sex-specific in Hong Kong Chinese subjects. *Clinical Endocrinology,* **40,** 63-66.

Cayzer, I., Chalmers, S.R., Doniach, D., & Swana, G. (1978) An evaluation of two new haemagglutination tests for the rapid diagnosis of autoimmune thyroid diseases. *J. Clin. Pathol.,* **31**, 1147-1151.

Chiovata, L., Santini, F., Vitti, P., Bendinelli, G. & Pinchera, A. (1994) Appearance of thyroid stimulating antibody and Graves' disease after radioiodine therapy for toxic nodular goitre. *Clinical Endocrinology*, **40**, 803-806.

Clarke, W.L., Shaver, K.A., Bright, G.M., Rogol, A.D. & Nance, W.E. (1984) Autoimmunity in congenital rubella syndrome. *J. Pediatr.,* **104**, 370-373.

Coyle, P.V., Wyatt, D., Connolly, J.H. & O'Brien, C. (1989) Epstein-Barr virus, infection and thyroid dysfunction. *Lancet*, **ii**, 899.

Creagh, F.M., Parkes, A.B., Lee, A., Adams, H., Hall., R., Richards, C.J. & Lazurus, J.H. (1994) The iodide perchlorate discharge test in women with previous post-partum thyroiditis: relationship to sonographic appearance and thyroid function. *Clinical Endocrinology,* **40**, 765-768.

Doble, N.D., Banga, J.P., Pope, R., Lalor, E., Kilduff, P. & McGregor, A.M. (1988) Autoantibodies to the thyroid microsomal/thyroid peroxidase antigen are polyclonal and directed to several distinct antigenic sites. *Immunol.,* **64**, 23-29.

Doerge, D.R. & Divi, R.L. (1995) Porphyrin pi-cation and protein radicals in peroxidase catalysis and inhibition by anti-thyroid chemicals. *Xenobiotica*, **25:7**, 761-767.

Doniach, D. & Roitt, I.M. (1957) Autoimmunity in Hashimoto's disease and its implications. *J. Clin. Endocrinol. Metab.*, **17**, 1293-1304.

Doniach, D., (1967) Thyroid autoimmune disease. *J. Clin. Path.*, **20**: 385-390.

Doniach, D., Bottazzo, F. & Drexhage, H.A. (1982) 'The autoimmune endocrinopthies'. In, Clinical Aspects of Immunology. (Lachmann, P.J., Peters, K., eds.). pp. 903-937. Blackwell Scientific Publications, Oxford.

Edwards, R. (1985). 'Immunoassay An Introduction'. William Heinemann Medical Books, London.

Engval, E. & Perlmann, P.J. (1972) *J. Immunol.*, **109**, 129. (Taken from Voller, Bidwell & Bartlett, 1979).

Ewins, D.L., Rosser, M.N., Butler, J., Roques, P.K., Mullan, M.J. & McGregor, A.M. (1991) Association between autoimmune thyroid disease and familial Alzheimer's disease. *Clinical Endocrinology (Oxf)*, **35**, 93-96.

Fan, J-L., Desai, R.K., Dallas, J.S., Wagle, N.M., Seetharamiah, G.S. & Prabhakar, B.S. (1994) High frequency of B cells capable of producing anti-thyrotropin receptor antibodies in patients with Graves' disease. *Clinical Immunology and Immunopathology*, **71:1**, 69-74.

Feldt-Rasmussen, U. (1996) Analytical and clinical performance goals for testing autoantibodies to thyroperoxidase, thyroglobulin and thyrotropin receptor. *CLIN. CHEM.*, **42:1**, 160-163.

Ferrari, C., Boghen, M., Paracchi, A., Rampini, P., Raiteri, F., Benco, R., Romussi, M. & Codecasa, F., Mucci, M. & Bianco, M. (1983) Thyroid autoimmunity in hyperprolactinaemic disorders. *Acta Endocrinologica*, **104**, 35-41.

Finke, R., Seto, P. & Rapoport, B. (1990) Evidence for the highly conformational nature of the epitopes(s) on human thyroid peroxidase that are recognised by sera from patients with Hashimoto's thyroiditis. *J. Clin. Endocrinol. Metab.*, **71:7**, 53-59.

Fong, S., Tsoukas, C.D., Frinke, L. A., Lawrence, S.K., Holbrook, T.L., Vaughn, J.H. & Carson, D.A. (1981) Age associated changes in Epstein-Barr virus induced human lymphocyte autoantibody production. *Journal of Immunology*, **126**, 910-914.

Forbes, I.J., Roitt, M., Doniach, D. & Solomon, K. (1962) The thyroid cytotoxic autoantibody. *J. Clin. Invest.*, **41**, 996-1006.

Gerstein, H.C. (1990) How common is postpartum thyroiditis? *Archives of Internal Medicine*, **150**, 1397-1400.

Gorin, A. & Lamburg, B.A. (1975) Natural course of symptomless autoimmune thyroiditis. *Lancet*, **ii**, 1234-1239.

Groves, C.J., Howells, R.D., Williams, S., Darke, C. & Parkes, A.B. (1990) Primary standardisation for the ELISA of serum thyroperoxidase and thyroglobulin antibodies and their prevalence in a normal Welsh population. *J. Clin. Lab. Immunol.*, **32**, 147-151.

Guo, J., Rapoport, B. & McLachlan, S.M. (1997) Thyroid peroxidase autoantibodies of IgG class in thyroid autoimmunity. *Clinical Immunology and Immunopathology*, **82:2**, 157-162.

Hackett, E. & Beech, M. (1960) Thyroglobulin antibodies in patients without clinical disease of the thyroid gland. *Lancet*, **2**, 402-404.

Hamada, N., Jaeduck, N., Portmann, L., Ito, K. & DeGroot, L.J. (1987) Antibodies against denatured and reduced thyroid microsomal antigen in autoimmune thyroid disease. *J Clin. Endocrinol. Metab.*, **64**, 230-238.

Harchali, A.A., Montagne, P., Ruf, J., Cuillière, M.L., Bene, M.B., Faure, G. & Duheille, J. (1994) Microparticle-enhanced nephelometric immunoassay of anti-thyroid peroxidase autoantibodies in thyroid disorders. *CLIN. CHEM.*, **40:3**, 442-447.

Harris, B.B., Othman, S., Davies, J.A., Weppner, G.J., Richards, C.J., Hall, R. & McGregor, A.M. (1988) Postpartum thyroid dysfunction in Mid-Glamorgan. *British Medical Journal*, **296**, 241-244.

Harris, B.B., Othman, S., Davies, J.A., Weppener, G.J., Richards, C.J., Newcombe, R.G., Lazurus, J.H., Parkes, A.B. & Phillips, D.I.W. (1992) Association between post-partum thyroid dysfunction and thyroid antibodies and depression. *British Medical Journal,* **305**, 152-156.

Hawkes, R., Niday, E. & Gordon, J. (1982) A dot immuno-binding assay for monoclonal and other antibodies. *Anal. Biochem.*, **119**, 142-147.

Hawkings, B.R., Cheah, P.S., Dawkins, R.L., Whittingham, S., Burger, H.G., Patel, Y., Mackay, I.R. & Welborn, T.A. (1980). Diagnostic significance of thyroid microsomal antibodies in a randomly selected population. *Lancet*, **ii**, 1057-1059.

Heidelberger, M. & Kendell, F.P. (1935) The precipitin reaction between type III pneumococcus polysaccharide and homologous antibodies. *J. Exp. Med.*, **61**, 563-591.

Helfand, M & Crapo, L.M. (1990) Screening for thyroid disease. *Ann. Intern. Med.*, **112**, 840-849.

Hennemann, G., Doctor, R. & Krenning, E.P. (1994) 'Thyroid hormone production, transport and metabolism' in Diseases of the Thyroid Pathophysiology and Management, 1st Edn. (Wheeler, M.H. & Lazurus, J.H., eds.), pp. 21-28, Chapman and Hall, London.

Hidaka, Y., Tamaki, H., Iwatani, Y., Tada, H., Mitsuda, N. & Amino, N. (1994) Prediction of post-partum onset of Graves' thyrotoxicosis by measurement of thyroid-stimulating antibody in early pregnancy. *Clinical Endocrinology*, **41**, 15-20.

Higashi, H., Omori, A. & Yamagata, T. (1992) Calmodulin, a ganglioside-binding protein. Binding of gangliosides to calmodulin in the presence of calcium. *J. Biol. Chem.*, **267**, 9831-9838.

Hijmans, W., Radl, J., Bottazzo, G.F. & Doniach, D. (1984) Autoantibodies in highly aged humans. *Mech. Aging Dev.*, **26**, 83-89.

Janeway, C.A. & Travers, P. (1996) Immunobiology: The Immune System in Health and Disease, 2nd Edn., Current Biology Ltd., London.

Jansson, R., Bernander, S., Karlsson, A., Levin, K. & Nilsson, G. (1984) Autoimmune thyroid dysfunction in the post-partum period. *J. Clin. Endocrinol. Metab.*, **58**, 681-687.

Jansson, R., Karlsson, A. & Dahlberg, P.A. (1985) Thyroxine methimazole and thyroid microsomal autoantibody titres in hypothyroid Hashimoto's thyroiditis. *British Medical Journal*, **290**, 11-12.

Jansson, R., Dahlberg, P.A. & Karlsson, A.F. (1988) 'Postpartum thyroiditis' in *Clinical Endocrinology and Metabolism: Hypothyroidism and Goitre*, **Vol 2** (Lazurus, J.H. & Hall, R., eds.), pp. 619-635, Balliére-Tindall, London.

Jokiranta, T.S. & Meri, S. (1993) Biotinylation of monolonal antibodies prevents their ability to activate the classical pathway of complement. *J. Immunol.*, **151**, 2124-2131.

Jones, R. & Payne, B. (1997) 'Clinical Investigation and Statistics in Laboratory Medicine', (Jones, G.J. & Payne, R.B., eds.), pp. 27-64, ACB Venture Publications, Piggott Printers (Cambridge Ltd.,).

Kanner, A.D., Coyne, J.C., Schaefer, C. & Lazurus, R.S. (1981) Comparison of two modes of stress measurement: Daily hassles and uplifts versus major life events. *J. Behav. Med.*, **4:1**, 1-39.

Kasagi, K., Konishi, J., Iida, Y., Tokuda, Y., Arai, K., Endo, K. & Torizuka, K. (1987) A sensitive and practical assay for thyroid-stimulating antibodies using FRTL-5 thyroid cells. *Acta Endocrinologica (Copenh.)*, **115**, 30-36.

Khoury, E.L., Hammond, L., Bottazzo, G.F. & Doniach, D.D. (1981) Presence of organic specific 'microsomal' autoantigen on the surface of human thyroid cells in culture: Its involvement with complement-mediated cytotoxicity. *Exp. Immunol.*, **45**: 316-328.

Khoury, E.L., Bottazzo, G.F. & Roitt, I.M. (1984) The thyroid "microsomal" antibody revisited. *J. Exp. Med.*, **159**, 577-591.

Kohno, Y., Hiyama, Y., Shimojo, N., Niimi, H., Nakajima, H. & Hosaya, T. (1986) Autoantobodies to thyroid peroxidase in patients with chronic thyroiditis: effect of antibody binding on enzyme activities. *Clin. Exp. Immunol.*, **65**, 534-541.

Kohno, Y., Naito, N., Hiyama, Y., Shimojo, N., Suzuki, N., Tarutani, O., Niimi, H., Nakajima, H. & Hosoya, T. (1988) Thyroglobulin and thyroid peroxidase share common epitopes recognized by autoantibodies in patients with chronic autoimmune thyroiditis. *J. Clin. Endocrinol. Metab.*, **67**, 899-907.

Kotani, T., Umeki, K., Matsunaga, S., Kato, E. & Ohtaki, S. (1986) Detection of autoantibodies to thyroid peroxidase in autoimmune thyroid disease by micro-ELISA and immunoblotting. *J. Clin. Endocrinol. Metab.*, **61**, 928-933.

Kricka, L.J. (1991) Chemiluminescent and bioluminescent techniques. *CLIN.CHEM.*, **37:9**, 1472-1481.

Kung, A.W.C. (1995) Life events, daily stresses and coping in patients with Graves' disease. *Clinical Endocrinology*, **42**, 303-308.

Lazurus, J.H., McGregor, A.M., Ludgate, M., Darke, C., Creagh, F.M. & Kingswood, C.J. (1986) Effect of lithium carbonate therapy on thyroid immune status in manic depressive patients: a prospective study. *Journal of Affective Disorders,* **11**, 155-160.

Lazurus, J.H. & Othman, S. (1991) Thyroid disease in relation to pregnancy. *Clinical Endocrinology*, **34**, 91-98.

Lazurus, J.H. (1996) Investigation and treatment of hypothyroidism. *Clinical Endocrinology,* **44**, 129-131.

Leclere, J. & Weryha, G. (1989) Stress and auto-immune endocrine diseases. *Horm. Res.*, **31**, 90-93.

Lee, S., Chow, C.C., Wing, Y.W. & Shek, C.C. (1992) Thyroid abnormalities during chronic lithium treatment in Hong Kong Chinese: A controlled study. *Journal of Affective Disorders*, **26**, 173-178.

Leri, O., Sinpoli, M.T., Di Prima, M.A. & Paggi, A. (1992) Hepatic C virus antibodies and Graves' disease. *British Medical Journal,* **January 14**, 310-311.

Ludgate, M., Mariotti, S., Libert, F., Dinsart, C., Piccolo, P., Santini, F., Ruf, J., Pinchera, A. & Vassart, G. (1989) Antibodies to human thyroid peroxidase in autoimmune thyroid disease: Studies with a cloned recombinant complementary deoxyribonucleic acid epitope. *J. Clin. Endocrinol. Metab.*, **68:6**, 1091-1096.

Mariotti, S., Anelli, S., Ruf, J., Bechi, R., Czarnocka, B., Lombardi, A., Caryon, P. & Pinchera, A. (1987) Comparison of serum thyroid microsomal and thyroid peroxidase autoantibodies in thyroid diseases. *J. Clin. Endocrinol. Metab.*, **65:5**, 987-993.

Mariotti, S., Caturegli, P., Piccolo, P., Barbesino, G. & Pinchera, A. (1990) Antithyroid peroxidase autoantibodies in thyroid diseases. *J. Clin. Endocrinol. Metab.*, **71:3**, 661-669.

Mariotti, S., Francheschi, C., Cossarizza, A. & Pinchera, A. (1995) The Aging Thyroid. *Endocrine Reviews,* **16:6,** 686-715.

Massart, C., Guilhem, I., Gibassier, J., Allannic, H. & Nicol, M. (1991) Comparison of thyroperoxidase and microsomal antibody asays in sera from patients with Graves' disease. *CLIN. CHEM.*, **37:10**, 1777-1780.

McClachlan, S.M. & Rapoport, B. (1995) Genetic and epitope analysis of thyroid peroxidase (TPO) autoantibodies: markers of the human thyroid autoimmune response. *Clin. Exp. Immunol.*, **101**, 200-206.

Miles, J., Charles, P. & Riches, P. (1998) A review of methods available for the identification of both organ-specific and non-organ-specific autoantibodies. *Ann. Clin. Biochem.,* **35**, 19-47.

Miralles, F., Takeda, Y. & Escribano, M.J. (1991) Comparison of carbohydrate and peptide biotinylation on the immunological activity of IgG1 murine monoclonal antibodies. *J. Immunol. Meths.*, **140**, 191-196.

Morita, S., Tetsuhiko, A. & Midori, M. (1995) Prevalence of nonthyroid specific autoantibodies in autoimmune thyroid disease. *J. Clin. Endocrinol. Metab.*, 1202-1206.

Mukata, T., Tamai, H., Oshima, A., Morita, T., Matsubayashi, S., Fukata, S. & Kuma, K. (1994) Immunological findings and thyroid function of untreated Graves' disease patients with undetectable TSH-binding inhibitor immunoglobulin. *Clinical Endocrinology*, **40**, 215-219.

Muzykantov, V.R., Gavriluk, V.D., Reineeke, A., Atochina, E.N., Kuo, A., Barnathan, E.S. & Fisher, A.B. (1995) The functional effects of biotinylation of anti-angiotensin-converting enzyme monoclonal antibody in terms of targeting in vivo. *Anal. Biochem.*, **226**, 279-287.

Nakajima, Y., Howells, R.D., Pegg, C., Davies-Jones, E. & Rees-Smith, B. (1987) Structure-activity analysis of microsomal antigen/thyroid peroxidase. *Mol. Cell. Endocrinol.*, **53**, 15-23.

Nelson, N. C. & Kacian, D.L. (1990) Chemiluminescent DNA probes: a comparison of the acridinium ester and dioxetane detection systems and their use in clinical diagnostic assays. *Clin. Chim. Acta., (Elsevier),* **194**, 73-90.

Ng, M.L., Rajna, A. & Khalid, B.A.K. (1987) Enzyme immunoassay for simultaneous measurement of autoantibodies against thyroglobulin and thyroid microsome in serum. *CLIN. CHEM.*, **33:12**, 2286-2288.

Nicholson, L.B., Wong, F.S., Ewins, D.L., Butler, J., Holland, A., Demaine, A.G. & McGregor, A. M. (1994) Susceptibility to autoimmune thyroiditis in Down's syndrome is associated with the major histocompatibility class II DQA 0301 allele. *Clinical Endocrinology*, **41**, 381-383.

Nystrom, E., Caidahl, K., Fager, G., Wikkelso, C., Lundberg, P. & Lindstedt, G. (1988) A double-blind cross-over 12 month study of L-thyroxine treatment of women with 'subclinical' hypothyroidism. *Clinical Endocrinology,* **29**, 36-76.

O'Connor, G. & Davies, T.F. (1990) Human autoimmune thyroid disease. A mechanistic update. *TEM* May/June, 266-274.

Othman, S., Phillips, D.I.W., Parkes, A.B., Richards, C.J., Harris, B., Fung, H., Darke, C., John, R., Hall, R. & Lazurus, J.H. (1990) A long-term follow-up of post-partum thyroiditis. *Clinical Endocrinology*, **32**, 559-564.

Paggi, A., Caccavo, D., Ferri, G.M. Di-Prima, M.A., Amorosa, A., Vaccaro, F., Bonomo, L. & Afeltra, A. (1994) Anti-cardiolipin antibodies in autoimmune thyroid disease. *Clinical Endocrinology*, **40**, 3329-3333.

Parkes, A.B., McLachlan, S.M., Bird, P. & Rees Smith, B. (1984) The distribution of microsomal and thyroglobulin antibody activity among the IgG subclasses. *Clin. Exp. Immunol.*, **57**, 239-243.

Parkes, A.B., Williams, S., Howells, R.D., Harris, R., Lazurus, J.H., Waters, J.S., & Hall, R. (1991) The measurement of complement fixation by autoantibodies directed against thyroid membrane antigens. *J. Clin. Lab. Immunol.,* **35**, 1-7.

Parkes, A.B., Richards, C.J., Hall, R. & Lazurus, J.H. (1993) Complement C3 activation by thyroid peroxidase antibodies (TPOAb) and the pathogenesis of post-partum thyroid disease (PPT). *J. Endocrinol. Invest.,* **16**, 28.

Parkes, A.B., Black, E.G., Adams, H., John, R., Richards, C.J., Hall, R. & Lazurus, J.H. (1994) Serum thyroglobulin: an early indicator of autoimmune post-partum thyroiditis. *Clinical Endocrinology,* **41**, 9-14.

Parlett, G.R. (1978) 'Spectrophotometry' in Scientific Foundations of Clinical Biochemistry: Analytical Aspects, **Vol 1**. (Williams, D.L., Nunn, R.F. & Marks, V., eds.). pp. 55-72, William Heinemann Medical Books Limited, London.

Paschke, R., Vogg, M., Swillens, S. & Usadel, K.H. (1993) Correlation of microsomal antibodies with the intensity of the intrathyroidal autoimmune process in Graves' disease. *J. Clin. Endocrinol. Metab.*, **77:4**, 939-943.

Pharmacia Fine Chemicals (1983). 'Affinity Chromatography', Ch. 2., (Handbook).

Pop, V.J., De Vries, E., Van Baar, A.L., Waelkens, J.J., De Rooy, H.A., Horsten, M., Donkers, M.M., Komproe, I.H., Van Son, M.M. & Vader, H,.L. (1995) Maternal Thyroid Peroxidase Antibodies during Pregnancy: A Marker of Impaired Child Development?. *J. Clin. Endocrinol. Metab.*, **80:12**, 3561-3565.

Portmann, L., Hamada, N., Heinrich, G. & DeGroot, L.J. (1985) Anti-thyroid antibody in patients with autoimmune thyroid disease: possible identity with anti-microsomal antibody. *J. Clin. Endocrinol. Metab.*, **61**, 1001-1003.

Potter, K.N., Li, Y, & Capra, J.D. (1996) Staphylococcal Protein A simultaneously interacts with framework region 1, complementarity-determining region 2 and framework region 3 on human V_H3-encoded Igs[1]. *The Journal of Immunology*, 2982-2988.

Premawardhana, L.D.K.E., Lo, S.S.S., Phillips, D.I.W., Prentice, L.M. & Rees-Smith, B. (1994) Variability of serum thyroglobulin levels is determined by a major gene. *Clinical Endocrinology,* **41**, 725-729.

Prentice, L.M., Phillips, D.I.W., Sarsero, D., Beever, K., McLachlan, S.M. & Rees-Smith., B. (1990) Geographical distribution of subclinical autoimmune thyroid disease in Britain: A study using highly sensitive direct assays for autoantibodies to thyroglobulin and thyroid peroxidase. *Acta Endocrinologica (Copenh)*, **123**, 493-498.

Prud'homme G.J., Parfrey, N.A. & Vanier, L.E. (1991) Cyclosporin-induced autoimmunity and immunehypersensitivity. *Autoimmunity*, **9**, 345-356.

Rang, H.P., Dale, M.M. & Ritter, J.M. (1995) Pharmacology 3rd Edn., Churchill Livingstone, New York.

Rieu, M., Richard, A., Rosilio, M., Laplanche, S., Ropion, V., Fombeur, J.P. & Berrod, J.L. (1994) Effects of thyroid status on thyroid status on thyroid autoimmunity expression in euthyroid and hypothyroid patients with Hashimoto's thyroiditis. *Clinical Endocrinology,* **40**, 529-535.

Riley, W.J. (1995) Enzymes as antigens in autoimmune endocrinopathies. *CLIN. CHEM.*, **41:3**, 337-339.

Roitt, I.M., Doniach, D., Campbell, P.N. & Hudson, R.V. (1956) Autoantibodies in Hashimoto's disease (lymphadenoid goitre). *Lancet*, **ii**, 820-821.

Roitt, I.M. & Doniach, D. (1958) Human auto-immune thyroiditis: Serological studies. *Lancet*, **ii, November 15**, 1027-1033.

Roitt, I.M., Ling, N.R., Doniach, D. & Couchman, K.G. (1964) The cytoplasmic auto-antigen of the human thyroid immunological and biochemical characteristics. *Immunology*, **7**, 375-393.

Roitt, I.M. ed. (1996) Immunology, 5th Edn., Blackwell Scientific Publications, London.

Roman, S.H., Korn, F. & Davies, T.F. (1984) Enzyme-linked immunosorbent microassay and haemagglutination compared for the detection of thyroglobulin and thyroid microsomal autoantibodies. *CLIN. CHEM.*, **30:2**, 246-251.

Roti, E., Gardini, E., Minelli, R., Bianconi, L. & Braverman, L. E. (1992) Prevalence of anti-thyroid peroxidase antibodies in serum in the elderly: Comparison with other tests for anti-thyroid antibodies. *CLIN. CHEM.*, **38:1**, 88-92.

Ruf, J., Carayon, P. & Lissitsky, S. (1985) Various expression of a unique anti-human thyroglobulin antibody repertoire in normal state and autoimmune disease. *Eur. J. Immunol.*, **15**, 268-272.

Ruf, J., Czarnocka, M., Ferrand, M., Doullais, F. & Caryon, P. (1988) Novel routine assay of thyroperoxidase autoantibodies. *CLIN. CHEM.*, **34:11**, 2231-2234.

Ruf, J., Toubert, M.E., Czarnocka, B., Durand-Gorde, J.M., Ferrand, M. & Caryon, P. (1989) Relationship between immunological structure and biochemical properties of human thyroid peroxidase. *Endocrinology*, **125**, 1211-1218.

Ruf, J., Ferrand, M., Durand-Gorde, J.M., De-Micco, C. & Caryon, P. (1993) Significance of thyroglobulin antibodies cross-reactive with thyroperoxidase (TGPO antibodies) in individual patients and immunised mice. *Clin. Exp. Immunol.*, **92**, 65-72.

Schardt, C.W., McLachlan, S..M., Matheson, J. & Rees-Smith, B. (1982) An enzyme-linked immunoassay for thyroid microsomal antibodies. *J. Immunol. Meths.*, **55**, 155-168.

Steinman, L. (1995) Escape from "Horror Autotoxicus" : Pathogenesis and treatment of autoimmune disease. *Cell*, **80**, 7-10.

Storm, D., Loos, M. & Kaul, M. (1996) Biotinylation of proteins via amino groups can induce binding to U937 cells, HL-60 cells, monocytes and granulocytes. *J. Immunolog. Meth.*, **199**, 87-99.

Sundbeck, G., Eden, S., Jagenburg, R., Lundberg, P. & Lindstedt, G. (1995) Prevalence of serum antithyroid peroxidase antibodies in 85 year-old women and men. *CLIN. CHEM.*, **41:5**, 707-712.

Takusu, N., Yamada, T., Takusu, M., Komiya, I., Nagasawa, Y., Asawa, T., Shinoda, T., Aizawa, T. & Koizumi, Y. (1992) Disappearance of thyrotropin-blocking antibodies and spontaneous recovery from hypothyroidism in autoimmune thyroiditis. *New England of Journal Medicine*, **326**, 513-518.

Tomer, Y. (1997) Short Analytical Review - Anti-thyroglobulin autoantibodies in autoimmune thyroid disease: Cross-reactive or pathogenic? *Clinical Immunology and Immunopathology*, **82:1**, 3-11.

Trotter, W.R., Belyavin, G. & Waddams, A. (1957) Precipitating and complement-fixing antibodies in Hashimoto's disease. *Proc. Roy. Soc. Med.*, **50**, 961-964.

Tunbridge, W.M.G., Evered, D.C., Hall, R., Appleton, D., Brewis, M., Clark, F., Grimley Evans, J., Young, E., Bird, T. & Smith, P.A. (1977) The spectrum of thyroid disease in a community: the Wickham survey. *Clinical Endocrinology*, **7**, 481-493.

Vanderpump, P.J., Tunbridge, W.M.G., French, J.M., Appleton, D., Bates, D., Clark, F., Grimley-Evans, J., Hasan, D.M., Rodgers, H., Tunbridge, F. & Young, E.T. (1995) The incidence of thyroid disorders in the community: a twenty-year follow-up of the Wickham Survey. *Clinical Endocrinology*, **43**, 55-68.

Vanderpump, M.P.J., Ahlquist, J.A.O., Franklyn, J.A, & Clayton, R.N. (1996) *British Medical Journal*, **313**, 539-544.

Van Weeman, B.K. & Schuurs, A.H.W.M. (1971). *FEBS.*, Letters, **15**, 232 and **24**, 77. (Taken from Voller, Bidwell & Bartlett, 1979).

Voller, A., Bidwell, D.E, & Bartlett, A. (1979) 'The Enzyme-linked immunosorbent assay (ELISA): A guide with abstracts of microplate application'. Nuffield Laboratories of Comparative Medicine, The Zoological Society of London, Regent's Park, London. (Sponsored by Dynatech Europe).

Voller, A., Bidwell, D.E. & Burek, C.L. (1981) An enzyme-linked immunosorbent assay (ELISA) for antibodies to thyroglobulin. *Proc. Soc. Exp. Biol. Med.*, **163**, 402-405.

Volpé, R. (1989a) 'Autoimmune thyroiditis' in Thyroid Function and Disease, 1st Edn., (Burrow, G.N., Oppenheimer, J.H. & Volpe, R, eds.). pp. 191-207, WB Saunders Company, Philadelphia.

Volpé, R. (1989b) 'Graves' disease' in Thyroid Function and Disease, 1st Edn., (Burrow, G.N., Oppenheimer, J.H. & Volpe, R., eds.). pp. 214-269, WB Saunders Company, Philadelphia.

Volpé, R. (1994) Autoimmune endocrinopathies: aspects of pathogenesis and the role of immune assays in investigation and management. *CLIN. CHEM.*, **40:11(B)**, 2132-2145.

Weeks, I., Beheshti, I., McCapra, F., Campbell, A.K. & Woodhead, J.S. (1983) Acridinium esters as high-specific-activity labels in immunoassay. *CLIN. CHEM.*, **29:8**, 1474-1479.

Weetman, A.P., Rennie, D.P., Hassman, R., Hall, R. & McGregor, A.M. (1983) Enzyme-linked immunoassay of monoclonal and serum microsomal antibodies. *Clin. Chim. Acta.*, **138**, 237-244.

Weetman, A.P. & McGregor, A.M. (1984) Autoimmune thyroid disease: development in our understanding. *Endocr. Rev.*, **5**, 309-355.

Weetman, A.P., Nutman, T.B., Burman, K.D., Baker, J.R. & Volkman, D.J. (1987) Heterogeneity of thyroid autoantigens identified by immunoblotting. *Clin. Immunol. Immunopath.*, **43**, 333-342.

Weetman, A.P., Black, C.M., Cohen, C.B., Tomlinson, R.W.S., Banga, J.P & Reimer, C.B. (1989) Affinity purification og IgG subclasses and the distribution of thyroid auto-antibody reactivity in Hashimoto's thyroiditis. *Scand. J. Immunol.*, **30**, 73-82.

Weetman, A.P. & Borysiewicz, L.K. (1990) Viruses and autoimmunity. *Autoimmunity*, **5**, 277-292.

Weetman, A.P. (1991) 'Autoimmune Endocrine Disease'. Cambridge University Press, Cambridge.

Weetman, A.P. (1992) Autoimune thyroiditis: predisposition and pathogenesis. *Clinical Endocrinology*, **36**, 307-323.

Weetman, A.P. (1994) Prediction of post-partum thyroiditis. *Clinical Endocrinology*, **41**, 7-8.

Weetman, A.P. & McGregor, A.M. (1994) Autoimmune thyroid disease: Further developments in our understanding. *Endocrine Reviews*, **15:6**, 788-823.

Weetman, A.P. (1997) Hypothyroidism: screening and subclinical disease. *British Medical Journal*, **314**, 1175-1178.

Weetman, A.P. (2000) Subclinical thyroid disease. *Proceedings of Pathology 2000*, 57-58.

Weiner, H.L., Friedman, A., Miller, A., Khoury, S.J., Al-Sabbagh, A., Santos, L., Sayegh, M., Nussenblatt, R.B., Trentham, D.E. & Hafler, D.A. (1994) Oral Tolerance: Immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu. Rev. Immunol.*, **12**, 809-837.

Weiss., M., Ingbar, S.H., Winbald, S. & Kasper, D.L. (1983) Demonstration of a saturable binding site for thyrotropin in *Yersinia enterocolitica*. *Science*, **219**, 1331-1333.

Westphal, S. A. (1994) Seasonal variation in the diagnosis of Graves' disease. *Clinical Endocrinology*, **41**, 27-30.

Williams, D.L. & Goodburn, R. (1983) 'The Thyroid Gland' in Scientific Foundations of Clinical Biochemistry: Biochemistry in Clinical Practice, **Vol 2**. (Williams, D.L. & Marks, V., eds.). pp. 560-583, William Heinemann Medical Books Limited, London.

Wilson, & Nakane, P.K. (1978) Immunofluorescence and related techniques. Edn. Knapp, W. *et al.*, p.215, Elsevier, Holland. (Taken from Voller, Bidwell & Bartlett, 1979).

Yokoyama, N. & Taurog, A. (1988) Porcine thyroid peroxidase: relationship between the native enzyme and an active, highly purified tryptic fragment. *Mol. Endocrinol.*, **2**, 838-844.

Yokoyama, N., Taurog, A. & Klee, G.G. (1989) Thyroid peroxidase and thyroid microsomal autoantibodies. *J. Clin. Endocrinol. Metab.*, **68:4**, 766-773.

Yoshida, H., Amino, N., Yagawa, K., Uemura, K., Satoh, M., Miyai, K. & Kumahara, Y. (1978) Association of serum antithyroid antibodies with lymphocytic infiltration of the thyroid gland: studies of seventy autopsied cases. *J. Clin. Endocrinol. Metab.*, **46:6**, 859-862.

Yoshikawa, N., Arreaza, G., Mukata, T., Resetkova, E., Miller, N., Jamieson, C., Nishikawa,M., Inada, M. & Volpé, R. (1994) Effect of FK-506 on xenografted human Graves' thyroid tissue in severe combined immunodeficient mice. *Clinical Endocrinology*, **41**, 31-39.

Youde, S.J. & Parkes, A.B. (1996a) Stress proteins and thyroid autoimmunity - Evidence for common epitopes on both TPO and thyroid hsp70. *J. Endocrinol.*, **148**, **March** Supplement.

Zilva, J.F. & Pannall, P.R. (1984) Clinical Chemistry in Diagnosis and Treatment, 4th Edn., p 179. Lloyd-Luke (Medical Books) Ltd., London.

Zweig, M.H. & Campbell, G. (1993) Receiver-Operating Characteristics (ROC): a fundamental evaluation tool in clinical medicine. *CLIN. CHEM.*, **39:4**, 561-577.

APPENDICES

Appendix 1

All reagents used in the following appendix were obtained from BDH, unless otherwise stated in the text.

(1) Reagents, Buffers for Immunoradiometric Assay (Method 1)

(i) Tris HCL, pH 7.5 (Diluent for reconstituting the tracer and samples as described by Beever *et al.*, 1989)

150 mmol/l NaCl

10 mmol/l Tris HCl

5g of BSA (Bovine serum albumin) (Sigma-Aldrich Co. Ltd.)

1 ml of Tween 20

The NaCl and Tris (hydroxymethyl-methylamine) were weighed out and dissolved in approximately 600 cm^3 double-distilled water (ddH_2O) and the pH adjusted to 7.5 using concentrated HCl. BSA (5g) and 1 ml of the Tween 20 were added and the solution made up to 1 litre with ddH_2O.

(ii) 200 mmol/l Phosphate Buffer, pH 7.4

To 161 ml of 0.2M disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), a solution of 0.2M sodium dihydrogen phosphate (NaH_2PO_4) was added until pH 7.4.

(iii) 100 mmol/l Phosphate Buffer, pH 7.4

The 200 mmol/l phosphate buffer, pH 7.4 was diluted with an equal volume of ddH_2O.

(iv) 0.2M Sodium dihydrogen phosphate (NaH_2PO_4) (Mr=156.01)

15.601 g in 500 ml of ddH_2O.

(v) 0.2M Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) (Mr = 358.15)

35.815 g in 500 ml of ddH_2O.

(vi) 5% Glutaraldehyde (BDH)

3 ml of stock (5%) diluted with 12 ml of ddH_2O.

(vii) Sigma Protein A (extracellular) *Staphylococcus aureus* (Cat N° P6031)

Supplied as 5g of freeze-dried material.

(2) Reagents for Enzyme-immunometric Assay Using TPO Labelled with Horseradish Peroxidase (HRP) (Method 2)

(i) Acetate Buffer, pH 4.4

Anhydrous sodium acetate (8.2 g) was dissolved in approximately 100 ml of ddH_2O. Glacial acetic acid was added until pH was 4.4. (Acetate buffer diluted 10-fold with ddH_2O for dialysis).

(ii) Sodium Deoxycholate (20%)

4 g dissolved in 20 ml of ddH_2O.

(iii) Carbonate Buffer, pH 9.5

Sodium carbonate (0.5M) and sodium bicarbonate (0.5M) were prepared:-

Sodium carbonate (0.5M) (1.2 ml) was added to 10 ml of ddH_20 and 0.5 ml of 20% deoxycholate solution. The pH was adjusted to 9.5 with 0.5M sodium bicarbonate and made up to 25 ml with ddH_2O.

(iv) Sodium Periodate (0.1M)

Sodium periodate (0.0214 g) was dissolved in 1ml of ddH_2O - prepared freshly just before use.

(v) Phosphate-buffered Saline (PBS), pH 7.4

0.5M disodium hydrogen phosphate (35.49 g/500 ml)

0.5M sodium dihydrogen phosphate (39.00 g/500 ml)

Di-sodium hydrogen phosphate (0.5M) (32.1 ml), 18 g of sodium chloride, plus 4 ml of warmed 20% sodium deoxycholate was added to a beaker and the pH adjusted to 7.4 with sodium dihydrogen phosphate (0.5M). Then, 20 ml of 1% thiomersal was added and the solution made up to 2 litres with ddH_2O.

(vi) OPD Substrate (Voller, *et al.*, 1979)

25 ml of citrate-phosphate buffer (see vii)
1 OPD (ortho-phenylene diamine dihydrochloride) tablet (Sigma-Aldrich Co. Ltd.)
10 µl of hydrogen peroxide (H_2O_2)

(vii) Citrate/Phosphate Buffer, pH 5.0

24.3 ml of 0.1M citric acid (2.1014g /100 ml)
25.7 ml of 0.2M phosphate (7.163g $Na_2HPO_4.12H_2O$/100ml)
50 ml of ddH_2O

(3) Reagents for Chemiluminescent Assay [Method 3 (System 1)]

(i) Labelling Buffer [Phosphate Buffer (0.1M)], pH 8.0

15.6 g of $NaH_2PO_4.2H_2O$ per litre of ddH_2O.

(ii) Quenching Buffer

Lysine monohydrochloride (10 mg/ml)- supplied with kit and reconstituted with 5 ml of ddH_2O.

(iii) Bovine Serum Albumin (BSA)/Phosphate Mix (1% BSA Solution)

0.11 g BSA in 11 ml of 0.1M phosphate buffer, pH 8.0.

(iv) Elution Buffer (with 0.1% BSA)

10 ml of (iii) was added to 90 ml of (i) plus sodium azide (200mg/L).

(v) Diluent (for Stds, Controls and Tests) (PBS)

See Appendix 1 (5) for ELISA Diluent (PBS, pH 7.4).

(vi) ELISA Wash

See Appendix 1 (5) for ELISA Wash.

(4) Reagents Used in Method 3 (System 2)

Reagents for ECL Biotinylation Procedure

(i) Phosphate Buffered Saline, pH 7.5

11.5 g di-sodium hydrogen orthophosphate (anhydrous) (80mM)
2.96 g sodium dihydrogen orthophosphate (20mM)
5.84 g sodium chloride (100mM)

- the above were dissolved in approximately 800 ml of ddH_2O, the pH adjusted to 7.5 and the solution made up to a final volume of 1 litre.

(ii) Bicarbonate Buffer (40mM), pH 8.6

Stock 800mM supplied with kit, diluted 1 in 20 for use with ddH_2O.

(iii) Wash Reagent 2 : 0.1M Acetate Buffer, 0.5M NaCl, pH4.0

Sodium acetate (4.101 g) and sodium chloride (14.61 g) were dissolved in approximately 400 ml of ddH_2O, the solution was adjusted to pH 4.0 with HCl, and then made up to a final volume of 500 ml.

Reagents for CNBr-activated Sepharose™ 4B

Affinity Chromatography

(i) Coupling Buffer

$NaHCO_3$ (0.1M), NaCl (0.5M), pH 8.3 - requires to be freshly prepared.

(ii) Tris-Buffered Azide, pH 7.4

10 mM Tris

150 mM NaCl

200 mg/l Sodium Azide

5 ml of 2M Tris, 9g of NaCl and 200 mg of sodium azide were dissolved in 800 ml of ddH_2O, the pH was adjusted to 7.4 and the solution made up to 1 litre. (TBS, pH 7.4 also used with 1% BSA in the dilution of the Biotin-TPO label).

(iii) 2M Sodium Chloride

58.44 g NaCl in 500 ml of ddH_2O.

(iv) 3M Sodium Thiocyanate (NaSCN)

2.43 g sodium thiocyanate (anhydrous) in 10 ml of TBS/Azide, pH 7.4.

(5) Reagents for ELISA

(i) Coating Buffer

4.2 g of sodium bicarbonate

0.2g of sodium azide

-800 ml of ddH_2O was added, the pH adjusted to 9.3 and the solution made up to 1 litre with ddH_2O.

(ii) ELISA Wash

87.66 g NaCl

24.33 g Tris (Hydroxymethyl methylamine)

5 ml Tween Detergent

Approximately 600 ml of ddH_2O was added to the above, the pH adjusted to 7.4 and then made up to a final volume of 10 litres with ddH_2O.

(iii) Diluent (PBS) Phosphate-Buffered Saline, pH 7.4

1.56 g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)

(Mr=156 g)

9 g NaCl

0.2 g sodium azide

0.5 ml Tween

The above were dissolved in approximately 800 ml of ddH_2O, the pH adjusted to 7.4 and then made up to 1 litre with ddH_2O.

(iv) Antibody/Conjugate Reagent

25 ml of 0.9% saline (isotonic)

0.5 ml of sheep serum

25 µl of anti-human IgG_{FC} Peroxidase

(v) Substrate

0.01 g ABTS (2-2¹ Azino-di-(3-ethylbenzothiazolin sulphone-6 diammonium salt)

25 ml citrate phosphate buffer

10 µl hydrogen peroxide (H_2O_2)

(vi) Citrate Phosphate Buffer, pH 5.0

5.106 g Citric Acid

7.31 g Anhydrous disodium hydrogen phosphate (Na_2PO_4)

The above were dissolved in 800 ml of ddH_2O, the pH adjusted to 5.0 and then the solution made up to a final volume of 1 litre.

(vii) Stopping Reagent [can use either (a) or (b)]

(a) 0.04 g sodium fluoride (NaF) in 20 ml of ddH2O

(b) 10.5 g citric acid (i.e. 0.1M) in 500 ml of ddH2O

0.05 g sodium azide (0.01%)

Appendix 2

(1) Further Information on BioMag® 4100 Magnetic Particles Used in the IRMA (Method 1)

BioMag® 4100 consisted of a suspension of magnetic iron oxide particles coated to provide primary amino groups for the covalent attachment of proteins. The oxide particles are supermagnetic, i.e. they respond well to weak magnetic fields but do not become permanently magnetised. The amino groups are sterically unencumbered, permitting the covalent attachment of proteins or ligands (e.g. Protein A), with the retention of biological activity, (with a claimed coupling efficiency of 80% or greater).

BioMag® 4100 was supplied in distilled water containing 1mM solution EDTA (ethylene diamine tetra-acetate), pH 7.0, at a concentration of approximately 50 mg/ml, with about 12 μmoles of amine per ml in the BioMag® 4100 suspension as supplied (240 μmoles/g). Magnetic separations are superior to conventional separations when biohazardous or radioactive materials are involved. Separation of BioMag® from solution via a magnetic rack eliminated the need for centrifugation, which eliminated the possibility of aerosol production, inherent in centrifugation and also the possible contamination of equipment was minimised. Further details can be found in the product literature from Advanced Magnetics Inc., [see Appendix 4 for address].

(2) Micro Method for the Determination of Protein in Cerebrospinal Fluid and Urine

(a) Reagents

(i) Stock Ponceau S

0.4 g Ponceau S (BDH 'Gurr') in 19 ml of ddH_2O.

(ii) Stock TCA/Ponceau S

Trichloracetic acid (TCA) (30 g) was dissolved in 80 ml of ddH_2O, and 2 ml of stock ponceau S (reagent 1) was added and then made up to 100 ml with ddH_2O.

(iii) Working TCA/Ponceau S

Stock TCA/Ponceau S (reagent 2) (10 ml) was diluted to 100 ml with ddH_2O.

(iv) 0.2M Sodium Hydroxide (NaOH)

8g in a litre of ddH_2O.

(b) Method

The method was linear up to 2 g/l, and any proteins with a greater protein content required dilution with ddH_2O.

1. 100 µl of each supernatant, standard and control was pipetted into a plastic tube and 1 ml of the working TCA/Ponceau S solution was added to each tube, mixed well and centrifuged at full speed for 10 minutes.

2. The supernatant was then decanted without disturbing the precipitate and the tubes drained for 1 minute.

3. The precipitate was redissolved in 2.0 ml of 0.2M sodium hydroxide.

4. The absorbances were read at 560 nm using water as a blank and then the protein concentration was calculated in g/L using the known protein content of the standard preparation.

The standard and quality control used in the assay procedure were in current routine laboratory use, to ensure that the procedure was acceptable.

(3) Estimation of Immunoglobulin G (IgG)

The immunoglobulin G to be measured in the supernatants, was mixed with its specific anti-sera (Dako Ltd) and the resulting turbidity measured photocolorimetrically on the Cobas Fara II centrifugal analyser at 340 nm. A standard curve was produced using SPS-01 Calibrant, obtained from The Royal Hampshire Hospital, Sheffield, with Behring N/T Protein Control Serum used as quality control.

(4) Preparation of Thyroid Microsomes (as described by *Groves et al.*, 1990)

Thyroid microsomes (containing the crude TPO antigen) were prepared from thyroid tissue obtained during sub-total thyroidectomy for primary hyperthyroidism [Graves' disease (G.D.)] The tissue was dissected and snap frozen in hexane, cooled over alcohol/CO_2 and subsequently stored at minus 80°C. Tissue was minced in 0.15M KCl using a Polytron homogenizer (type PT350D, Northern Media Supplies Ltd., England) at full speed for 60 seconds. The homogenate was centrifuged at 10,000 g for 15 minutes at 4°C to remove cell debris before being recentrifuged (100,000; 60 minutes; 4°C). The pellet from this centrifugation, which represented a 'microsomal' fraction, was washed twice in phosphate buffered saline (PBS) (100,000 g; 4°C; 60 minutes) and suspended in 0.25M sucrose and solubilised using 10 volumes 0.1% sodium deoxycholate in 0.01M Tris, pH 8.0; insoluble material was removed by centrifugation (10,000g; 15 minutes; 4°C). The protein concentration of this soluble membrane fraction at this stage was found to be approximately 1 mg/ml using Folin phenol reagent. Human thyroglobulin (hTg) was isolated from the supernatant from the first $6x10^{-6}$ g/minute spin by taking an ammonium sulphate cut between 1.5M and 1.7M which was dialysed and purified on a column of Sephacryl S-300 equilibrated with Tris-buffered saline pH 7.0. Test analyses were carried out to determine the viability of the preparations for use in assays.

Appropriate precautions were taken in accordance with 'Health and Safety' guidelines for the handling of human tissue samples.

(5) Preparation of Magnetic Particles Coupled to Anti-Human Immunoglobulin (IgG) (MAb-Anti-IgG)

(i) Preparation of IgG Protein

1. The antibody [sheep anti-human IgG (Heavy & Light Chain Specific)] obtained from the Scottish Antibody Production Unit, was reconstituted in Acetate Buffer, pH 5.5 (as described in Appendix 1:2 but different pH).

2. Sodium sulphate was slowly added to give a final concentration of 180 mg/ml and mixed for 30 minutes at room temperature.

3. The mixture was centrifuged at 2000 r.p.m. for 20 minutes.

4. The pellet was redissolved in 4 ml of acetate buffer and dialysed 3 times overnight using acetate buffer.

(ii) Coupling of IgG Protein to Magnetic Particles

1. The magnetic particles (obtained from Advanced Magnetics Inc.) were washed 3 times with methanol.

2. The particles were resuspended in approximately 25 ml of acetate buffer and shaken-mixed in a culture flask for 3 minutes, and the particles separated on a magnetic plate. The particles were then resuspended in 25 ml of acetate buffer and shaken-mixed. This was repeated 3 times.

3. The particles were then resuspended in approximately 8 ml of acetate buffer.

4. An equal volume of 12.5% glutaraldehyde was added and rotate-mixed for 3-4 hours. The particles were then washed 5 times each with 25 ml of acetate buffer.

5. The particles were resuspended in 5 ml of acetate buffer and 5 ml of the antibody preparation gently added [prepared as described previously in (i)].

6. The mixture was gently mixed overnight at room temperature.

7. The particles were washed with PBS, pH 7.4 (see ELISA Reagents: Appendix 1) and then washed with 1M NaCl. This wash procedure was repeated 3 times.

8. The particles were then resuspended in diluent (phosphate buffer, pH 7.4).

9. The magnetic particle suspension coupled to anti-IgG (46 mg/ml) was then heat stressed for 16 hours at 50°C and subsequently washed 3 times with 25 ml of diluent.

(6) (i) Preparation of Human Immunoglobulin (Ig) for 1 Labelling with AE for Method 3 (System 2)

Ammonium sulphate was added to a serum sample containing a high TPOAb level to give a final concentration of 1.7M. This caused the Ig (containing high levels of IgG) to precipitate when left for 1 hour at 0°C, which was subsequently isolated by centrifugation at >2000g. The resulting pellet was washed with 1.6M ammonium sulphate. The final pellet was dissolved in 1/5 volume of distilled water before being dialysed against three changes of Tris buffered saline [see Reagents (ii) next]. The optical density at 280 nm was measured and the protein concentration calculated using the extinction coefficient:-

$$\sum_{1cm}^{280} = 1.4$$

The protein aliquots were stored at -70°C.

(ii) Reagents for Ammonium Sulphate Precipitation

Tris-Buffered Azide, pH 7.4

10 mM Tris

150 mM NaCl

200 mg/l Sodium Azide

To 5 ml of 2M Tris, 9g of NaCl and 200 mg of sodium azide was added and dissolved in 800 ml of ddH_2O. The pH was adjusted to 7.4 and the solution made up to 1 litre.

(7) Enzyme-linked Immunosorbent Assay (ELISA)

Each well of a (96-well) polystyrene, microtitre immunoassay plate (Dynatech Laboratories, INC.), was coated with 100 µl of human thyroid microsomes (10 µg/ml- diluted 1:100 in coating buffer, 50 mM sodium carbonate, pH 9.1) to give a well concentration of 1 µg/ml. [The MIC preparation was solubilised with 1% deoxycholate (DOC) in phosphate buffered saline (PBS)]. The plates were incubated overnight at 4°C and then decanted vigorously. Each plate was washed four times with ELISA Wash Solution [see Appendix 1:5] and drained thoroughly between each wash.

Standards (diluted as for Method 1 (System 3); quality control and samples (diluted 1:100); and blanks (PBS buffer, pH 7.4), (100 µl of each) were added to each well (loaded vertically in quadruplicate). The plates were incubated at room temperature for 2 hours then thoroughly washed again four times with ELISA Wash.

The plates were treated with peroxidase conjugated sheep anti-human IgG antibody (Serotec) (100 µl of freshly-prepared conjugate being added to each well). The plate was then incubated for a further half hour at room temperature and the washing procedure repeated again. The autoantibody activity was quantified following the addition of ABTS (2,2¹-Azino-bis(3-ethylbenzthiozoline-6-sulphonic acid)- [Boerhinger) and hydrogen peroxide as substrate reagent (with 100 µl of freshly prepared substrate being added to each well).

The plate was read at intervals at 410 nm using the 'Dynatec' Multi-Plate Reader, with the colour measured spectrophotometrically at 492 nm. When the optical density of the top standard reached 1.00, 50 µl of stopping reagent was added across the plate. The final optical densities were then measured and the logarithmically converted standard data was plotted, and the test absorbances

interpolated using the Olivetti M24 microcomputer using 'in-house' software written in 'Turbo Pascal'.

(8) Indirect Agglutination

The data for the indirect agglutination assays in this project, were provided using the Serodia®-AMC kit for the detection and titration of thyroid microsomal antibodies in human serum. This method utilised artificial gelatin particle carriers 'sensitised' with thyroid microsomal antigen, extracted and purified from human thyroid tissue. The coloured, artificial gelatin carriers are claimed to eliminate every possible chance for the production of 'non-specific'-agglutination associated with passive haemagglutination (PH), and be easier to read than their PH counterpart (which utilised 'erthrocytes sensitised' with thyroid microsomes as the carriers). In addition, results were provided in 3 hours and the final patterns were stable overnight.

Assay Procedure

Serum samples (appropriately diluted with kit diluent) were incubated with 'sensitised' particles in the 'U'-shaped wells of a (96-well) micro-titre. The contents of the wells were thoroughly mixed and allowed to stand on a level surface, at room temperature for 3 hours and the patterns interpreted. 'Unsensitised' particles were also incorporated into the assay to demonstrate any effects of 'non-specific' agglutination, together with a positive control serum.

Appendix 3

Affinity Chromatography

Affinity chromatography enables the purification of almost any biomolecule on the basis of its biological function or individual chemical structure. Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilised on an insoluble support (matrix).

It is important that the immobilised ligand retains its specific binding affinity for the substance of interest and that methods are available for selectively desorbing the bound substances in an active form, after washing away unbound material.

Sepharose® is a bead-formed agarose gel which displays virtually all the features required of a successful matrix for immobilising biologically active molecules. The hydroxyl groups on the sugar residues can be easily derivatised for covalent attachment of a ligand. Sepharose® 4B is the most favoured and widely-used matrix. The open-pore structure, and the exclusion limit of Sepharose® 4B in gel filtration is MWt $20x10^{-6}$, makes the interior of the matrix available for ligand attachment and ensures good binding capacities, even for large molecules. Sepharose® 4B exhibits extremely low non-specific adsorption; this is essential because the power of affinity chromatography relies on specific interactions.

In 1972 Pharmacia Fine Chemicals developed CNBr-activated Sepharose® 4B, a stable ready-to-use medium for immobilising ligands. CNBr-activated Sepharose® 4B enables ligands containing primary amino groups to be safely, easily and rapidly immobilised by a spontaneous reaction [see Figure (i) for structure].

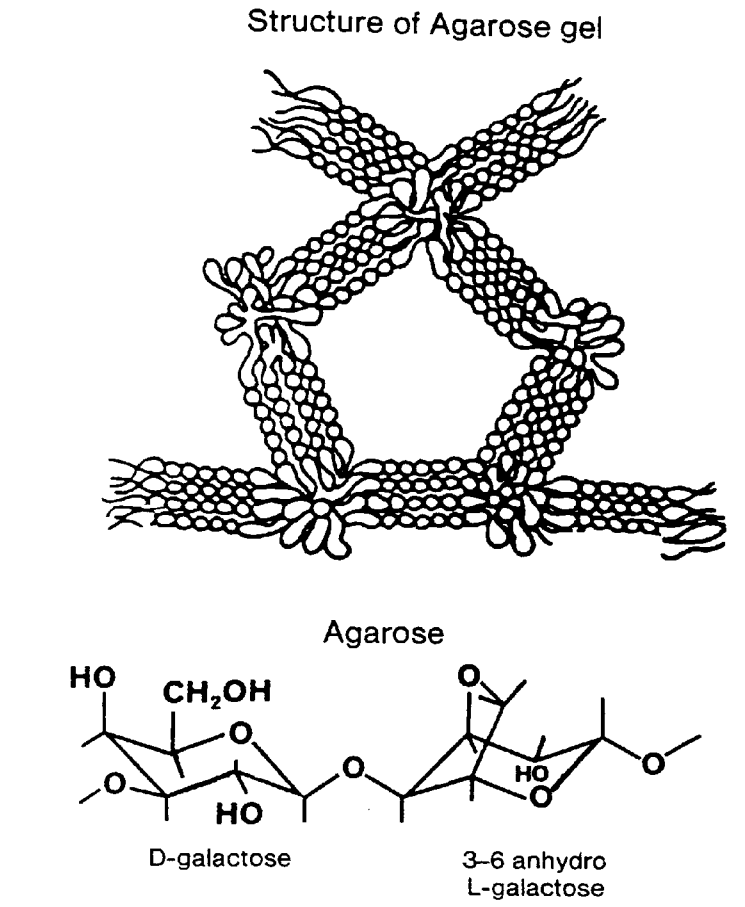


Figure (i) Structure of agarose gel.

Taken from Pharmacia Fine Chemicals (Handbook), 1983, (Ch.3).

The coupling reaction is spontaneous, rapid and easy to carry out and the amount of coupled ligand can be reproducibly controlled. Because cyanogen bromide, which is an extremely toxic and unpleasant chemical, is absent, the use of CNBr-activated Sepharose™ 4B requires no special chemicals or equipment.

CNBr-activated Sepharose™ 4B is produced by the reaction of Sepharose™ 4B with cyanogen bromide. The active product is freeze-dried in the presence of additives to preserve the bead form of the gel. The freeze-dried material (1g) swells to give approximately 3.5 ml of swollen gel. The freeze-dried material is supplied in packs of 15 g and should be stored below 8°C. Under these conditions the shelf life is approximately 18 months.

The selection of the ligand for affinity chromatography (i.e. TPO in this method) is influenced by two factors. Firstly, the ligand should exhibit specific and reversible binding affinity for the substance to be purified. Secondly, it should have chemically modifiable groups which allow it to be attached to the matrix without destroying its binding activity.

Preparation of CNBr-Activated Sepharose™ 4B Gel

CNBr-activated Sepharose™ 4B Gel (1g) was weighed out into an appropriate container (universal). Approximately 20 ml of 1mM HCl was added in order to wash and swell the gel. The solution was then filtered using a Buchner filter funnel system. The gel was washed quickly with a further 200 ml of 1mM HCl, with care being taken not to let the gel dry out, and then washed in a similar manner using 20 ml of coupling buffer ($NaHCO_3$ buffer, pH 8.3, containing 0.5M NaCl) [see Appendix 1:4].

Appendix 4

Suppliers: The following companies supplied equipment or consumables, as detailed in the text and used in this project.

Advanced Magnetics Inc., Metachem Diagnostics Ltd., 29, Forest Road, Piddington, Northampton, NN7 2DA.

Amersham LIFE SCIENCE, Amersham International plc., Amersham Place, Little Chalfont, Buckinghamshire, England, HP7 9NA.

Bayer plc (formerly Chiron Diagnostics), Registered Office Bayer House Strawberry Hill, Newbury, Berkshire, RG14 1JA.

Becton & Dickinson, Between Towns Road, Cowley, Oxford, OX4 3LY, England.

Boehringer Mannheim Ltd., (Diagnostics and Chemicals), Bell Lane, Lewes, East Sussex, BN7 1LG, England.

B.D.H. (British Drug House Ltd.), Poole, England.

Cambridge Life Sciences plc., Cambridgeshire Business Park, Angel Drove, Ely, Cambridge, CB7 4DT, England.

Behring (now Dade-Behring), Walton Manor, Walton, Milton Keynes, Buckinghamshire, MK7 7AJ.

Dynal (UK) Ltd., 10, Thursby Road, Croft Business Park, Bromborough, Wirral, Merseyside, L62 3PW.

Dynatech Laboratories, INC., 14340 Sullyfield Circle, Chantilly, Virginia, 22021.

Fujirebio INC. (Serodia® AMC), 7-1, Nishi-shinjuku 2-chome, Shinjuku-ku, Tokyo, Japan.

Medicell International Ltd., 239, Liverpool Road, London, N1 1LX

Molecular Light Technology Research Ltd., Cardiff Business Technology Centre, Senghenydd Rd., Cardiff, CF2 4AY.

National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom.

Pharmacia LKB Biotechnology (Fine Chemicals), 23, Grovener Road, St. Albans, Hertfordshire, AL1 3AW, England.

R.S.R. Ltd., Avenue Park, Pentwyn, Cardiff, U.K.

Sarstedt Ltd., 68, Boston Road, Beaumont Leys, Leicester, LE4 1AW, England.

Serotec U.K., 22, Bankside, Station Approach, Kidlington, Oxford, OX5 1JE, England.

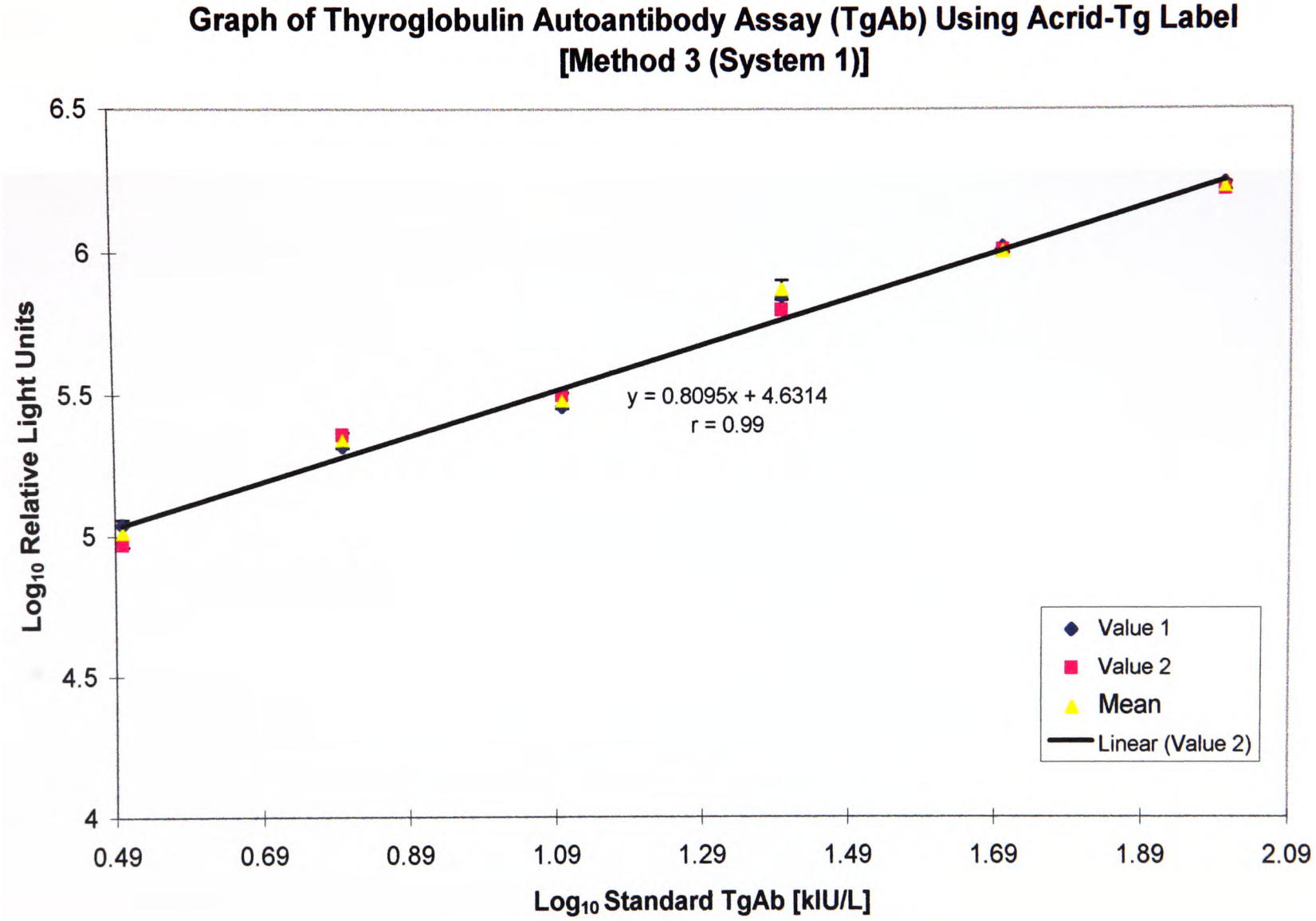
Sigma-Aldrich Co. Ltd., Fancy Road, Poole, Dorset, HB17 7NH, U.K.

Appendix 5

The following graph is of a thyroglobulin autoantibody (TgAb) assay using the assay system [Method 3 (System 1)] [see 2.6] as described for the measurement of thyroid peroxidase autoantibodies (TPOAb) in human serum.

A preparation of Tg was labelled with AE using the same procedure employed in the preparation of Acrid-TPO (using the 'Molecular Light' labelling kit). The Tg used in the label was prepared at a different stage within the same procedure as used for the preparation of the thyroid microsomes (TPO) (Groves *et al.*, 1990) [see Appendix 2:4]. The appropriate molar concentration (i.e. 0.3 nmol of Tg) was used with 5 μg of AE in the preparation of the Acrid-Tg label. The standards (but with a different dilution regime), quality control and assay procedure was as used in the TPOAb assay.

A viable assay system was produced with a 17% uptake of AE (an order of 10^6 RLUs) exhibited by the top standard as compared with the meagre uptake of <2% (an order of 10^4 RLUs) in the assays using Acrid-TPO (label prepared using 5.5 μg of acridinium ester). Very good assay precision was demonstrated, with a correlation coefficient (r) of 0.99 produced [see Graph next page, error bars defined as 1 S.D.].


Graph of Thyroglobulin Autoantibody Assay (TgAb) Using Acrid-Tg Label
[Method 3 (System 1)]
6.5
6
5.5
5
4.5
4
Log10 Relative Light Units
y = 0.8095x + 4.6314
r = 0.99
Value 1
Value 2
Mean
Linear (Value 2)
0.49
0.69
0.89
1.09
1.29
1.49
1.69
1.89
2.09
Log10 Standard TgAb [kIU/L]